

**THE ROLE OF TNF-RELATED
APOPTOSIS-INDUCING
LIGAND IN IMMUNE
FUNCTION**

PRESENTED BY

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"Be curious always! For knowledge will not acquire you: you must acquire it".

Sudie Black.

Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand [TRAIL or Apo-2 ligand (L)] is a member of the TNF superfamily of ligands that can induce cellular responses such as activation, proliferation, differentiation, migration and apoptosis. Recombinant (r) soluble TRAIL is currently being developed as a most promising natural immune molecule for trial in cancer patients since it selectively induces apoptosis in transformed or stressed cells, but not in most normal cells. Unlike TNF and FasL (CD95L), that both exert significant systemic toxicities, rTRAIL has been shown to be relatively non-toxic and to exert potent anti-tumor functions when administered *in vivo* to tumor-bearing mice and non-human primates. Moreover, whilst radiation and most deoxy ribonucleic acid (DNA)-damaging chemotherapeutic drugs induce tumor cell apoptosis in a p53-dependent manner, TRAIL-mediated apoptosis is p53-independent. Treatment with rTRAIL might therefore be expected to circumvent resistance to conventional chemotherapy and radiotherapy in cancer patients lacking p53 function.

Despite great interest in TRAIL as a cancer therapeutic, either as a sole agent or in combination with irradiation or chemotherapeutic drugs, what has undeniably been lacking is an in depth understanding of the natural physiological role of TRAIL. This knowledge is fundamental if TRAIL is to be used safely and with efficacy in the clinic. Very recently, constitutive TRAIL expression has been identified on a small subset of liver natural killer (NK) cells in adult mice. TRAIL expression can also be induced on interferon (IFN)- α -stimulated peripheral blood T cells, IFN-stimulated human monocytes and dendritic cells (DCs), and NK cells stimulated with interleukin (IL)-2, IFNs or IL-15. We and others have reported that TRAIL-expressing liver NK cells play a role in suppression of TRAIL-sensitive liver tumor metastasis *in vivo*, suggesting that TRAIL might play a role in tumor surveillance. In a small number of preliminary studies, TRAIL has also been identified to suppress autoimmune disease induction.

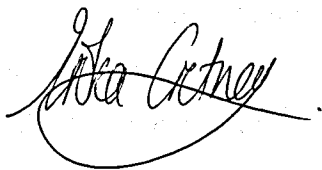
The focus of this thesis has been to help elucidate the roles of endogenous host TRAIL in disease. The research described herein provides the first detailed

evidence of the leukocyte expression and function of mouse TRAIL. We describe the initial characterization of TRAIL-deficient mice generated by gene-targeting, and use these mice to (1) identify TRAIL as a marker of NK cell differentiation, (2) determine the role for TRAIL in regulation of tumor development, (3) determine the role for TRAIL in T cell development and homeostasis, and (4) determine the importance of TRAIL in controlling the induction of experimental autoimmune encephalomyelitis (EAE).

DECLARATION

This is to certify that

- (i) the thesis comprises only my original work towards the PhD except where indicated in the acknowledgements,
- (ii) due acknowledgement has been made in the text to all other material used,
- (iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

A handwritten signature in black ink, appearing to read 'Erika Cretney', with a large, sweeping loop at the end.

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The data shown in Figures 4.1 and 4.2 of Chapter 4 was generated by Erika Cretney prior to undertaking PhD candidature.

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ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
α -GalCer	α -Galactosylceramide
AICD	Activation induced cell death
AIRE	Autoimmune regulator
ALPS	Autoimmune lymphoproliferative syndrome
APAF-1	Apoptotic protease-activating factor 1
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B-cell-activating receptor
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
BCG	Bacillus Calmette-Guerin
BCMA	B-cell maturation antigen
Bid	BH3-interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
Caspase	Cysteine protease
CDDP	cis-diaminedichloroplatinum (II)
CFA	Complete Freund's adjuvant
cFLIP	Cellular FLICE inhibitory protein
CLP	Common lymphoid progenitor
CMJ	Corticomedullary junction
CML	Chronic myelogenous leukaemia
Clr	c-type lectin related
CNS	Central nervous system
CO ₂	Carbon dioxide
CP	Chronic progressive
CR	Complete resistance

CRD	Cysteine-rich domain
cTEC	Cortical thymic epithelial cell
CTL	Cytotoxic T lymphocyte
CTLR	C-type lectin receptor
DC	Dendritic cell
DcR	Decoy receptor
DD	Death domain
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double negative
DNA	Deoxy ribonucleic acid
DP	Double positive
DR	Death receptor
ds	Double stranded
E	Embryo day
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDA	Ectodermal dysplasin
EDAR	Ectodyplasin-A-receptor
ELISA	Enzyme-linked immunosorbent assay
ENDO G	Endonuclease G
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ES	Embryonic stem cell
FADD	Fas-associated-death domain
Fas	CD95/Apo-1 receptor
FasL	Fas ligand (CD95 ligand)
FCS	Fetal calf serum
FHL	Familial haemophagocytic lymphohistiocytosis
FITC	Fluoroscein isothiocyanate
FLIP	FLICE inhibitory protein
FTOC	Fetal thymic organ culture
g	Gram

GC	Germinal centre
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR family receptor
<i>gld</i>	Generalized lymphoproliferative disease (FasL mutant)
GVL	Graft-versus-leukemia
GRB2	Growth-factor receptor-bound protein 2
Grz	Granzyme
h	Hour
H & E	Haematoxylin and eosin
HA	Haemagglutinin
HDAC7	Histone deacetylase 7
HLA	Human leukocyte antigen
HN	Haemagglutinin-neuraminidase
HSC	Haematopoietic stem cell
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
Ig	Immunoglobulin
I κ BNS	Inhibitor of nuclear transcription factor κ B
IL	Interleukin
iNKT	Invariant natural killer T
i.p.	Intraperitoneal
ITAM	Immunoregulatory tyrosine based activation motif
ITIM	Immunoregulatory tyrosine based inhibition motif
i.v.	Intravenous
JNK	JUN N-terminal kinase
KIR	Killer cell immunoglobulin-like receptor
L	Ligand
LAK	Lymphokine activated killer
LAT	Linker for activation of T cells
<i>lpr</i>	Lymphoproliferation (Fas receptor mutant)
lps	Lipopolysaccharide
LT	Lymphotoxin

LZ	Leucine zipper
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCA	Methylcholanthrene
MCMV	Murine cytomegalovirus
MEF2D	Myocyte enhancer factor 2D
μg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class-I-chain related protein
min.	Minute
μl	Microlitre
ml	Milliliter
μM	Micromolar
mm	Millimeter
mM	Millimolar
MMTV	Mouse mammary tumor virus
MMR	Mismatch-repair
MNC	Mononuclear cells
MOG	Myelin oligodendrocyte glycoprotein
Monokine	Monocyte-derived cytokine
MPR	Mannose phosphate receptor
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear transcription factor kappaB
NK	Natural killer
NKP	Natural killer cell precursor
NKT	Natural killer T cell
nm	Nanometer
nM	Nanomolar

NOD	Non-obese diabetic
Nor1	Neuron-derived orphan receptor 1
Nur77	Nuclear receptor 77
Ocl	Osteoclast inhibitory lectin
OPG	Osteoprotegerin
PCR	Polymerase chain reaction
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PE	Phytoerythrin
pfp	Perforin
pg	Picogram
pH	Negative decimal logarithm of hydrogen-ion in moles/litre
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PTK	Protein tyrosine kinase
PLP	Proteolipid protein
R	Receptor
r	Recombinant
Rae-1	Retinoic acid early transcript-1
RANK	Receptor activator of nuclear factor- κ B
RAR α	Retinoic-acid receptor α
RELT	Receptor expressed in lymphoid tissues
rpm	Revolutions per minute
RR	Relapsing-remitting
S	Short
SAP	Signaling lymphocyte activation molecule-associated protein
s.c.	Subcutaneous
SCZ	Subcapsular zone
Scid	Severe-combined immunodeficient
SEM	Standard error of the mean
SEB	<i>Staphylococcus</i> enterotoxin B
Serpin	Serine protease inhibitor
SP	Single positive

SR	Spontaneous resistance
TACI	Transmembrane activator and cyclophilin ligand interactor
TAP	Transporter associated with antigen processing
tBID	Truncated BH3-interacting domain death agonist
TCR	T cell receptor
6-TG	6-Thioguanine
THD	Tumor necrosis factor homology domain
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor-associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
ULBP	UL16-binding proteins
vFLIP	Viral FLICE inhibitory protein
WT	Wild-type
XEDAR	X-linked ectodermal dysplasia receptor
XLP	X-linked proliferative syndrome
ZAP-70	ξ -associated protein-70

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The following literature review provides an extensive insight into our current understanding of NK cell biology, with a specific focus on members of the TNF superfamily. These elements of the immune system have the capacity to maintain the immune system in addition to orchestrating and regulating innate and adaptive immunity, autoimmune disease and tumorigenesis. This review also details the promise of TRAIL as a potential cancer therapeutic and highlights our currently limited knowledge of the true physiological role of TRAIL, knowledge that is fundamental if the TRAIL pathway is to be manipulated safely and with efficacy in the clinic.

Understanding how NK cell function is regulated, the pathways used by NK cells to eliminate cancerous and virus-infected host cells, and the complex network of interactions between NK cells and other immune cells has been a great undertaking for NK cell biologists. The continued discovery of new members of the TNF superfamily, their important function in modulating both innate and adaptive immunity, and their anti-cancer potential has emphasized more than ever the critical need to understand the biological roles of these proteins.

The TNF superfamily has been described as representing a double-edged sword (Aggarwal, 2003), since while they are essential for normal physiological responses, inappropriate expression and function of some members can be harmful as illustrated in autoimmune disease (Desplat-Jego et al., 2002; Joosten et al., 1996; Maini et al., 1995; Williams et al., 1992). In an attempt to further understand the physiological role(s) of TRAIL, this thesis describes the first reported characterization of a TRAIL gene-targeted mouse by determining a role for TRAIL in the regulation of tumor development, homeostasis of the immune system, and control of autoimmune disease induction.

1.2 Natural Killer (NK) cells

NK cells are a unique subset of lymphocytes that represent a crucial component of the innate immune system. NK cells comprise ~5% of splenic lymphocytes, ~10% of liver lymphocytes, ~5-20% of PBL and are present at lower frequencies in the thymus, BM and lymph nodes (LNs). Whereas T cell development occurs in the thymus, the site of mainstream NK cell development in the adult is the BM (Lian and Kumar, 2002). NK cells lack rearranged receptors, but express a myriad of immunoglobulin (Ig)-like and C-type lectin receptors [CTLR(s)] that deliver a finely tuned balance of inhibitory and activating signals to regulate NK cell function (Borrego et al., 2002; Takei et al., 1997). A number of these receptors recognize transformed and virus-infected cells and can stimulate NK cell cytokine production, secretion of cytotoxic granules and expression of ligands that enable the NK cells to control virus infection and tumor initiation and spread.

A defining feature of NK cells is their ability to lyse tumor targets which lack class I MHC expression (Ljunggren and Karre, 1985; van den Broek et al., 1995) allowing them to often spontaneously kill cancerous or virus-infected host cells that have down-regulated class I MHC expression in order to evade the adaptive immune response (Algarra et al., 2000). Unlike T cells that require previous exposure to antigen in order to become activated, NK cells can mediate spontaneous killing of tumor cells and play an important role in the body's defense against microbial infection (Cerwenka and Lanier, 2001; Smyth et al., 2002b; Trinchieri, 1989; Trinchieri, 1995). NK cells, unlike T cells, are incapable of "memory" when they are exposed to tumor cells or virus-infected cells and therefore cannot generate an accelerated immune response, nor a larger number of effector cells on secondary exposure to the same tumor. In general, NK cells can only prevent the outgrowth of small numbers of tumor cells and are easily overwhelmed by larger challenges of tumor cells, thus presenting a problem in eliminating larger tumor masses (Wu and Lanier, 2003).

NK cells have a variety of effector mechanisms enabling them to mount a potent anti-tumor response. Although NK cells can mediate spontaneous killing of MHC class I-deficient tumor cells, resting NK cells in the periphery quickly become activated and proliferate upon stimulation with a variety of cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21, IFN- α and IFN- β . This is well documented in the host response to virus, when high levels of type I IFN are produced. NK cells can be exclusively responsible for rejecting tumors by directly killing tumor cells (Karre et al., 1986; van den Broek et al., 1995) or alternatively, they can stimulate components of the adaptive immune system to eliminate tumors (Kelly et al., 2002b; Wu et al., 1995). For example, NK cells may evoke T cell immunity against secondary MHC class I-sufficient tumors when primary rejection involves CD27 (Kelly et al., 2002a) or NKG2D (Diefenbach et al., 2001) expressed on NK cells. In addition, MHC class I^{low} tumors have been demonstrated to activate NK cells *in vivo* and subsequently prime DCs to induce protective CD8⁺ T cell responses (Mocikat et al., 2003). These links between NK cells and adaptive immunity remain poorly defined at a molecular level, but are likely important in anti-tumor responses. Activated NK cells themselves can also produce a variety of cytokines and chemokines that can have a direct effect on tumor growth and prime immune effector cells that are crucial for subsequent adaptive immune responses. NK cells use two major mechanisms to induce target cell apoptosis, the granule exocytosis pathway (Trapani et al., 2000; Trapani and Smyth, 2002) and the DR pathway (Nagata and Golstein, 1995; Smyth et al., 2003). Whilst the pfp/grz granule exocytosis pathway has been extensively studied, less is known about NK cell death ligand function.

1.2.1 NK cell development

NK cell development in the adult is thymus-independent and occurs in the BM (Lian and Kumar, 2002). During fetal development, hematopoietic stem cells (HSC) colonize the fetal liver before migrating to the BM where adult hematopoiesis occurs (Cumano and Godin, 2001). HSC give rise to a common lymphoid progenitor (CLP) population that can develop into NK, T and B cells (Kondo et al., 1997; MacDonald et al., 2001). A population of CD122⁺NK1.1⁺

DX5⁺ committed NK cell progenitors have been identified in adult BM by Rosmaraki and colleagues (Rosmaraki et al., 2001). These NK cell precursors (NKP) lack cytotoxic activity and transcripts for pfp and grzB, but can be stimulated to express NK1.1 and DX5 and gain cytotoxic function upon culture in high-dose IL-2. Importantly, NKP fail to develop into T or B cells, illustrating a unique commitment to the NK cell lineage. NK cell progenitors have also been identified in fetal thymus (Ikawa et al., 1999; Michie et al., 2000; Rodewald et al., 1992) and NK cells can develop in FTOC (Carlyle and Zuniga-Pflucker, 1998), although the relevance of this developmental pathway is unclear given that NK cell development is normal in athymic nude mice (Herberman et al., 1975).

IL-15 secreted by the BM stroma is essential for NK cell development in humans and in mice and plays a role in driving the final maturation process (Kennedy et al., 2000; Mrozek et al., 1996). IL-15-deficient mice have low numbers of BM NK1.1⁺ NK cells that are mature, cytotoxic and secrete cytokines (Colucci et al., 2003). Interaction between membrane-bound LT on NK cells with the LT- β receptor (LT β R) expressed on stromal elements is necessary to stimulate stromal cells to provide a microenvironment permissive for NK cell development (Iizuka et al., 1999; Wu et al., 2001). Although not essential, cytokines such as stem cell factor, FLT3 ligand and IL-7, can also promote NK cell development (Colucci et al., 2003; Lian and Kumar, 2002).

The cytokines that regulate specific developmental steps and the phenotype and functional characteristics of each intermediate towards NK cell maturation have been of great interest to NK cell biologists. Initially, researchers likened human NK cell development to that observed for T cells where CD4⁺ T helper cells differentiate into T_H1 (IFN- γ -producing) and T_H2 (IL-4-, IL-5- and IL-13-producing) subsets, driven by IL-12 and IL-4 respectively (Mosmann and Sad, 1996). Human NK cells were reported to differentiate into NK1 and NK2 subsets after culture in type 1 (rIL-12 + anti-IL-4 mAb) or type 2 (rIL-4 + anti-IL-12 mAb) inducing conditions (Peritt et al., 1998) (Figure 1.1A). Whilst the NK1 subset was shown to produce IFN- γ and IL-10, the NK2 subset produced IL-5 and IL-13 (Peritt et al., 1998) analogous to the T_H1 and T_H2 T cell subsets.

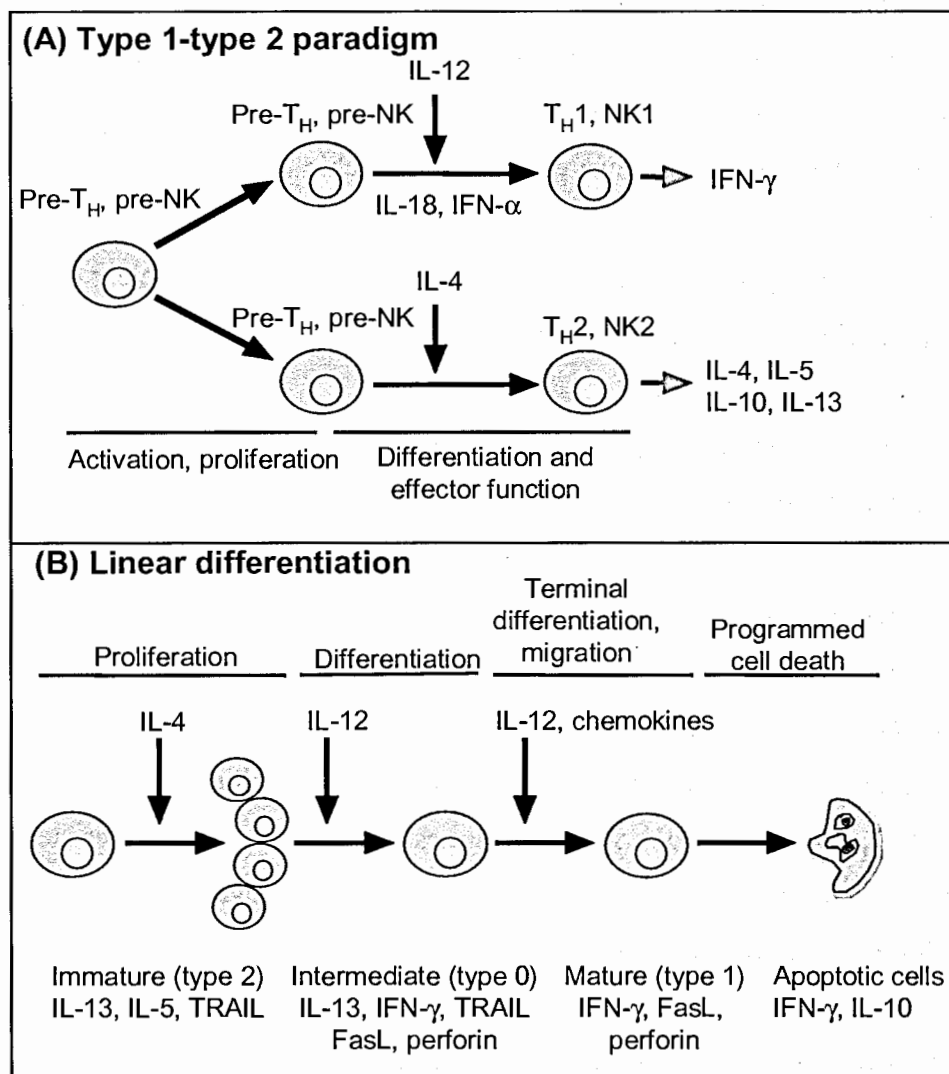


Figure 1.1. Progressive models of NK cell development. (A) Type 1-type 2 paradigm. Similar to the differentiation of T_H precursors (pre-T_H) into T_H1 and T_H2 subsets, NK cell precursors (pre-NK) were originally thought to differentiate into either type 1 (NK1) or type 2 (NK2) cytokine producing NK cells driven by IL-12 and IL-4 respectively. (B) Linear differentiation model. Immature, type 2 cytokine producing NK cells have now been shown to proliferate in the presence of IL-4 and differentiate into intermediate (type 0) NK cells when cultured in the presence of IL-12. Intermediate NK cells produce both IL-13 and IFN-γ and further differentiate into mature (type 1) NK cells upon stimulation with IL-12. Type 1 NK cells produce IFN-γ and are capable of pfp-mediated cytotoxicity. As NK cells differentiate, they lose TRAIL expression and acquire the ability to exert FasL- and pfp-dependent cytotoxicity. Adapted from (Colonna, 2001; Loza and Perussia, 2001).

Meanwhile, immature NK cells isolated from human umbilical cord blood were identified to be CD56⁺CD161⁺, to lack the IFN- γ transcript and to exert TRAIL- rather than pfp- and grz-mediated apoptosis (Bennett et al., 1996; Zamai et al., 1998). A similar population of immature NK cells was later identified in human PBL and demonstrated to secrete IL-5/IL-13 and to proliferate rapidly in response to IL-4 (Loza and Perussia, 2001). A linear differentiation pathway for human NK cell development has now been proposed by Perussia and colleagues (Loza and Perussia, 2001) (Figure 1.1B). The previously identified immature IL-13/IL-5-producing, TRAIL-expressing NK cells were defined as 'Immature' (type 2) and were revealed to proliferate rapidly in the presence of IL-4. Upon IL-12 stimulation, 'Immature' NK differentiate into 'Intermediate' (type 0) NK cells that can produce both IL-13 and IFN- γ and exert TRAIL-, FasL- and pfp-mediated cytotoxicity. IL-12 stimulated, 'Intermediate' NK cells further differentiate into 'Mature' (type 1) NK cells that produce IFN- γ and mediate apoptosis by FasL and pfp.

The individual stages of murine NK cell development have been identified on the basis of functional, phenotypic and proliferative capacities of NK cell developmental intermediates. Yokoyama and colleagues suggested five putative stages of NK cell maturation in mice (Kim et al., 2002) (Table 1.1). The earliest lineage-committed precursors (stage I) were characterized by expression of the IL-2 and IL-15 receptor common β subunit (IL-2-IL-15R β or CD122). This is followed by sequential acquisition of NK1.1 and CD94-NKG2 receptor expression and the integrin α_v subunit in stage II of maturation. Stage III NK cells gain Ly49 and c-Kit expression and this is followed by an NK cell expansion stage (IV) that is characterized by an upregulation of DX5 expression and downregulation of the integrin α_v subunit. During the final stage of NK cell maturation (stage V), NK cells upregulate Mac-1 and CD43 expression and mature functionally, producing high levels of IFN- γ and exerting high levels of cytotoxicity.

Stage	I	Acquisition of CD94- NKG2 and Ly49		Expansion stage IV	Mature NK cells V
		II	III		
Expression marker /function					
CD122	+	+	+	+	+
NK1.1	-	+	+	+	+
CD94-NKG2	-	+	+	+	+
Ly49	-	-	+	+	+
c-Kit	NA	-	+	+	+ / -
αv	NA	hi	hi	lo	-
DX5	NA	lo	lo	hi	hi
Mac-1	NA	lo	lo	lo	hi
CD43	NA	lo	lo	lo	hi
Cytotoxicity	NA	NA	NA	lo	hi
IFN- γ	NA	NA	NA	lo	hi

Table 1.1. Stages of *in vivo* murine NK cell development. BM NK cell developmental stages progress from the immature lineage-committed precursor (stage I) to mature NK cell (stage V). The presence (+) or absence (-) of various expression markers and NK cell function is noted at each stage of development. For some markers, expression is quantitated as high (hi) or low (lo). NA, not applicable. Adapted from (Kim et al., 2002).

1.2.2 Regulation of NK cell killing

NK cell function is controlled by a balance of NK cell inhibitory and activating signals (Figure 1.2). NK cell inhibitory receptors bind to self-MHC class I molecules, whereas NK cell activating receptors bind to ligands expressed on stressed, transformed, and virus-infected cells. Three inhibitory receptor families exist: the killer cell Ig-like receptors (KIRs) which are expressed in humans; the Ly-49 lectin-like homodimers, which are expressed in mice; and the CD94-NKG2A lectin-like receptors which are expressed in both mice and humans. Following NK cell inhibitory receptor ligation to MHC class I molecules, the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail of these receptors is phosphorylated at tyrosine residues, allowing recruitment of the tyrosine phosphatases SHP1 or SHP2, which deliver an inhibitory signal to the NK cell. A wide variety of NK cell activating receptors exist, many of these have short cytoplasmic domains and interact with transmembrane signaling adaptor molecules (DAP12, DAP10, FcR γ and CD3 ξ) to activate NK cell function. The cytoplasmic domains of these adaptor molecules contain immunoreceptor tyrosine based activating motifs (ITAMs) (Reth, 1989) or YxxN motifs, which upon stimulation, recruit ZAP70/SYK and phosphatidylinositol-3 kinase (PI3K) (Billadeau et al., 2003; Chang et al., 1999; Lanier et al., 1998; Wu et al., 1999b) respectively, delivering an activating signal to the NK cell. The NK cell activation state is therefore determined by the balance of positive and negative signals to the NK cell.

1.2.2.1 Classical major histocompatibility complex (MHC) class I receptors

A number of NK cell receptors primarily recognize classical MHC class I molecules. In humans, these include the KIRs and in mice the Ly49 receptors. To date, 14 KIRs have been described in humans as well as 2 non-expressed pseudogenes (Martin et al., 2000; Wilson et al., 2000). The majority of humans express between 6 and 14 of these genes (Uhrberg et al., 1997; Witt et al., 1999).

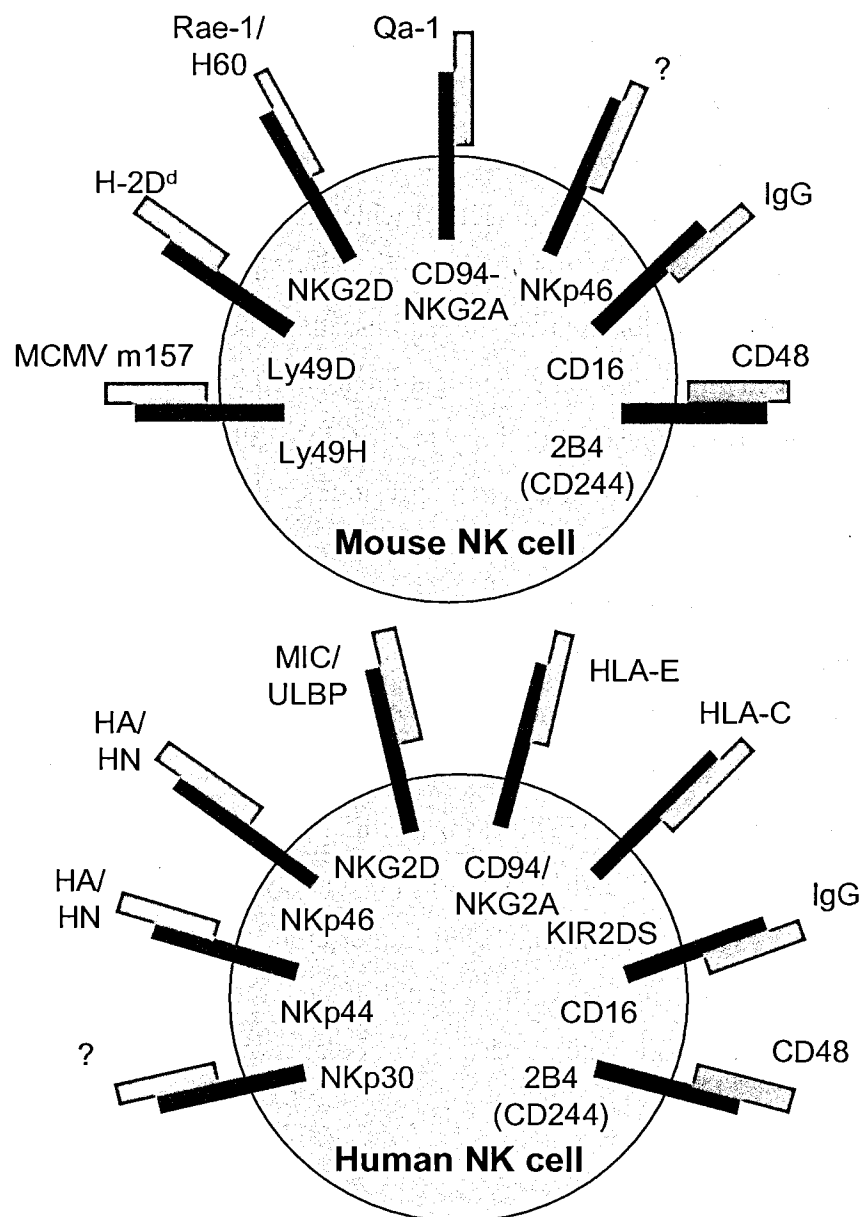


Figure 1.2. NK cell activating receptors and ligands. NK cell activating receptors play an important role in NK cell recognition and elimination of tumors and stressed cells. A wide variety of activating receptors exist, with some receptors (eg. NKG2D) expressed on the cell surface of both mouse and human NK cells. NK cell activating receptors are shown within the NK cell and NK-cell activating ligands are shown outside the NK cell. Abbreviations: HA, haemagglutinin; HN, haemagglutinin neuraminidase; HLA, human leukocyte antigen; MCMV, mouse cytomegalovirus; Rae-1, retinoic acid early transcript 1; MIC, MHC class-I-chain related protein; ULBP, UL16-binding protein.

At least 16 Ly49 genes and pseudogenes (Ly49A-Ly49Q) have been described in mice (Wilhelm et al., 2002).

The KIRs are type I transmembrane glycoproteins that contain 2 or 3 extracellular C2-type Ig domains (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995). Members of the KIR family share greater than 90% sequence identity and recognize polymorphic epitopes on the human MHC class I molecules HLA-A, HLA-B and HLA-C. Inhibitory KIRs contain ITIMs in their cytoplasmic tails, and activating KIRs possess a shorter cytoplasmic tail and a positively charged residue in the transmembrane region that allows them to interact with activating adaptor proteins (Lanier, 1998; Long, 1999).

The murine Ly49s are type II transmembrane glycoproteins and members of the CTLR superfamily. The Ly49s appear to be functionally equivalent to the human KIRs and recognize polymorphic epitopes on the murine MHC class I molecules H-2D and H-2K (Takei et al., 1997; Yokoyama et al., 1995). The majority of Ly49s are inhibitory receptors that contain ITIMs in their cytoplasmic tails (Brennan et al., 1996; Hanke et al., 1999; Karlhofer et al., 1992). Ly49D and Ly49H are activating receptors that associate with membrane-bound DAP12 to activate NK cells via the ZAP70/SYK pathway (Smith et al., 1998). Ly49C/I inhibitory receptor blockade augmented both *in vitro* NK cell cytotoxicity and *in vivo* anti-tumor activity (Koh et al., 2001) demonstrating the importance of inhibitory receptors for dampening the NK cell response.

1.2.2.2 Non-classical MHC class I receptors

The heterodimeric CD94/NKG2 receptors are conserved throughout the species and recognize the non-classical MHC class I molecules HLA-E (in humans) and Qa-1b (in rodents). Members of the NKG2 family are type II integral membrane proteins and require dimerization with CD94 glycoprotein for stable cell surface expression. HLA-E and Qa-1b bind to signal peptides derived from classical MHC class I molecules and their cell surface expression is transporter associated

with antigen processing (TAP)-1-dependent (Lee et al., 1998; O'Callaghan et al., 1998).

NKG2A is an inhibitory receptor that contains 2 cytoplasmic ITIMs that allow transmission of an NK cell inhibitory signal upon recognition of HLA-E or Qa-1b (Borrego et al., 1998; Braud et al., 1998; Vance et al., 1998). *In vivo*, growth of Qa-1b transfected TAP-deficient RMA-S tumor is increased compared with untransfected RMA-S, suggesting that NK cell activity is inhibited by CD94/NKG2A-Qa-1b interaction (Jia et al., 2000). NKG2C and NKG2E are activating receptors that associate with DAP12 by interaction with charged transmembrane residues (Yokoyama and Plougastel, 2003). This allows recruitment of ZAP-70/SYK and activation of a signal cascade that leads to NK cell activation.

1.2.2.3 NKG2D

NKG2D is a type II disulphide-linked dimer that contains a lectin-like extracellular domain but is only distantly related to the other NKG2 family members. Unlike other NKG2 receptors, NKG2D does not associate with CD94 but exists independently at the cell surface as a homodimer (Ding et al., 1999). In the mouse, NKG2D is expressed not only on freshly isolated NK cells, but also on lymphokine activated killer (LAK) cells, activated CD8⁺ T cells and macrophages as well as subsets of NKT and $\gamma\delta$ T cells (Jamieson et al., 2002; Diefenbach et al., 2000). Human NKG2D is expressed by most NK, CD8⁺ and $\gamma\delta$ T cells (Bauer et al., 1999).

NKG2D recognizes antigens expressed on stressed, virus- and bacteria-infected, abnormal or cancerous cells (Groh et al., 1998; Vetter et al., 2002). Human NKG2D recognizes MICA, MICB and the ULBPs 1-4 (Bauer et al., 1999; Cosman et al., 2001) whereas murine NKG2D recognizes the minor histocompatibility molecule H60, members of the Rae-1 family (Cerwenka et al., 2000; Diefenbach et al., 2001) and the ULBP-like molecule, MULT-1 (Carayannopoulos et al., 2002). *In vitro*, NKG2D ligand-expressing tumor cells

can stimulate nitric oxide synthesis and TNF- α transcription by activated murine macrophages (Diefenbach et al., 2000) and tumor cell lysis by human $\gamma\delta$ T cell clones is increased when these cells are cross-linked with anti-NKG2D mAb (Bauer et al., 1999). *In vivo* rejection of NKG2D ligand-expressing tumor cells is mediated by NK and CD8⁺ T cells (Cerwenka et al., 2001; Diefenbach et al., 2001), is largely pfp-dependent and appears independent of DR signaling or cytokine production (Hayakawa et al., 2002a).

NKG2D has been identified as an activating receptor that signals by associating with DAP10, thereby triggering the PI3K pathway (Wu et al., 1999b). In addition, NKG2D has been reported to act as a co-stimulatory molecule on T cells (Groh et al., 2001). These functional differences observed for NKG2D are possibly explained by the existence of two NKG2D isoforms [NKG2D-long (L) and NKG2D-short (S)] (Diefenbach et al., 2002). High levels of NKG2D-L messenger ribonucleic acid (mRNA) are found in freshly isolated naïve NK cells and in stimulated CD8⁺ T cells, whereas NKG2D-S mRNA is upregulated in activated NK cells and CD8⁺ T cells. Using immunoprecipitation studies, the same researchers demonstrated that while NKG2D associated with both DAP10 and DAP12 in activated NK cells, NKG2D only associated with DAP10 in activated CD8⁺ T cells. Moreover, DAP10 preferentially associated with NKG2D-L to mediate a co-stimulatory signal (as occurs in both NK and CD8⁺ T cells), whereas DAP12 associated with NKG2D-S to initiate an activating signal (as occurs in NK cells). These findings were supported by experiments undertaken by Colonna and colleagues who demonstrated a complete abrogation of NKG2D function in CD8⁺ T cells isolated from DAP10-deficient mice, but only partially impaired NKG2D function in activated NK cells (Gilfillan et al., 2002). In contrast, NKG2D in DAP12-deficient mice was functional in T cells, but impaired in NK cells (Diefenbach et al., 2000). These data demonstrate the importance of NKG2D signaling for the provision of both stimulatory and co-stimulatory signals to NK cells and a co-stimulatory signal in activated CD8⁺ T cells.

1.2.2.4 Non-MHC class I receptors

Although a number of NK cell receptors recognize and bind classical and non-classical MHC-class I molecules, members of the NKRP1 and natural cytotoxicity receptor (NCR) families of receptors bind non-MHC ligands such as c-type lectin related (Clr) molecules and viral proteins. Many of the ligands for these receptors remain undiscovered and the cellular ligands expressed by tumor cells are yet to be defined.

The NCRs NKp30 and NKp44 (in humans) and NKp46 (in mice and humans) are selectively expressed on NK cells. Whereas NKp46 and NKp30 are expressed on resting and activated NK cells, NKp44 is selectively expressed on activated NK cells. The NCRs are activating NK receptors that function by coupling with ITAM containing adaptor proteins such as DAP12 (for NKp44) or CD3 ζ and Fc ϵ RI γ (for NKp30 and NKp46) (Cantoni et al., 1999; Pessino et al., 1998). To date, the influenza protein HA and the HN protein of parainfluenza virus have been identified as ligands for NKp46 and NKp44, suggesting an important role for the NCRs in NK-mediated anti-viral responses (Arnon et al., 2001; Mandelboim et al., 2001). Tumor ligands for the NCRs are yet to be discovered, although a number of studies advocate that the NCRs play a role in tumor elimination. Firstly, a direct correlation exists between cell surface NCR expression on NK cells and the cytolytic capability of these cells in an anti-tumor response (Sivori et al., 1999). Secondly, blocking the function of one or more NCRs with specific antagonistic mAbs diminishes human NK cell killing of a number of human tumor cell lines (Moretta et al., 2000; Pende et al., 1999; Sivori et al., 1997; Vitale et al., 1998).

The NKRP1 family contains both activating and inhibitory receptors and bind to specific Clr molecules such as Clrb (osteoclast inhibitory lectin or Ocil) and Clrg. Clrg is predominantly expressed on DCs and macrophages (Carlyle et al., 2004; Iizuka et al., 2003). Clrb is expressed on the majority of haematopoietic cells excluding red blood cells and can inhibit NK cell killing when expressed on MHC class I-deficient tumor cells (Carlyle et al., 2004; Iizuka et al., 2003). A

single human member of the NKR1 family exists, NKR-P1A, which is expressed on a subset of mature NK cells and acts as an activatory receptor (Lanier et al., 1994). Four NKR1 receptors have been described in C57BL/6 mice, including NK1.1 (NKR1c), a common cell surface marker used to identify NK cells. NK1.1 associates with Fc ϵ RI γ , transmitting an activation signal to the NK cell (Arase et al., 1997). Similarly, NKR1a and NKR1f are thought to be activating receptors on account of the presence of a charged transmembrane residue in these receptors (Plougastel et al., 2001). The cytoplasmic tails of NKR1d and NKR1b (expressed in SJL/J and SWR mice) contain ITIM sequences and act as inhibitory receptors (Carlyle et al., 1999; Kung et al., 1999).

1.2.2.5 Co-stimulatory molecules

NK cell co-receptors interact with ligands expressed on target cells and provide co-stimulatory signals to the NK cell that range from enhancement of proliferation and cytokine production to the triggering of cytotoxicity. The role of the co-receptors is to amplify NK cell activation. Co-stimulatory molecules have been extensively characterized in T cells and demonstrated to be of great importance in the T cell anti-tumor response.

CD28 is an NK cell activation receptor that interacts with CD80 (B7-1) and CD86 (B7-2) on target cells and provides a co-stimulatory signal in both human and mouse NK cells (Galea-Lauri et al., 1999; Nandi et al., 1994; Wilson et al., 1999). Expression of CD80 in the highly metastatic mouse T lymphoma cell line BW-Li renders them more sensitive to NK cell-mediated cytotoxicity and decreases tumor metastases in mice (Geldhof et al., 1995). Similarly, CD80 expression on class I-deficient RMA-S tumor cells promoted NK cell-mediated tumor rejection and at higher tumor doses promoted both NK cell- and T cell-mediated tumor rejection (Kelly et al., 2002b). CD80 stimulated NK cell-mediated tumor rejection was not observed in IFN- γ ^{-/-} or pfp^{-/-} mice illustrating the importance of these molecules in the CD80-mediated tumor rejection process. Furthermore, NK cell-mediated rejection of RMA-S-CD80 stimulated tumor-specific T cell immunity against subsequent tumor challenge.

Another important NK cell co-stimulatory receptor is CD27 that is reportedly expressed by most murine NK cells and by a proportion of human NK cells (Sugita et al., 1992; Takeda et al., 2000b). CD27 is a member of the TNF superfamily of receptors and rather than triggering cytotoxicity plays an important role in the stimulation of NK cell proliferation and IFN- γ production (Takeda et al., 2000b). Expression of the CD27 ligand CD70 on MHC class I-deficient RMA-S tumor cells enhanced primary NK cell-mediated tumor rejection *in vivo* (Kelly et al., 2002a). Tumor rejection mediated by NK cells was IFN- γ - and pfp-dependent and evoked a secondary T cell-mediated immune response to parental MHC class I-sufficient RMA tumor (Kelly et al., 2002a). These key studies revealed the importance of CD27-CD70 interactions in providing a link between innate and adaptive immunity.

The NK cell co-receptor 2B4 (CD244) is a 70 kDa protein that binds CD48, a broadly expressed cell-surface glycoprotein. 2B4 serves a dual role and can transduce either an inhibitory or activating NK cell signal depending on expression of signaling lymphocyte activation molecule-associated protein (SAP) (Parolini et al., 2000), a downstream signaling molecule. Given sufficient SAP expression within the NK cell, 2B4 transduces an activating signal, but in the absence of SAP, SH2-domain containing protein tyrosine phosphatase-2 (SHP-2) binds 2B4 generating an inhibitory signal (Tangye et al., 1999). 2B4 is thought to co-operate with ITAM-containing NK cell receptors (Sivori et al., 2000) and to augment integrin-dependent NK cell activation (Barber and Long, 2003) via association with the cytoskeleton (Watzl and Long, 2003). Other reports suggest that 2B4 can trigger NK cell IFN- γ and IL-2 production as well as cytotoxicity (Garni-Wagner et al., 1993; Mathew et al., 1993). The importance of 2B4-mediated NK cell activation is clearly illustrated in patients with X-linked proliferative (XLP) syndrome (detailed in Chapter 1, Section 1.2.6).

1.2.2.6 Antibody-dependent cellular cytotoxicity (ADCC)

NK cells can be activated to induce ADCC by engagement of their low affinity cell surface Fc γ RIII receptors with the Fc portion of antibodies bound to target

cell-associated antigens. In humans, NK cell Fc γ RIIIA exists as a multimeric complex consisting of a ligand binding α subunit (CD16), which associates with homo- or hetero-dimers of γ or ξ (Anderson et al., 1989; Kurosaki et al., 1991; Kurosaki and Ravetch, 1989; Lanier et al., 1989). ITAMs in the γ and ξ subunits can recruit intracellular signaling molecules which ultimately lead to activation of the NK cell (Cambier, 1995; Reth, 1989). Murine Fc γ RIII receptors contain only γ - γ homodimers (Kurosaki and Ravetch, 1989) and NK cells from $\gamma^{-/-}$, but not $\xi^{-/-}$ mice lack the ability to mediate ADCC (Liu et al., 1993; Takai et al., 1994). Upon ligation of Fc γ RIII receptors, protein tyrosine kinases (PTKs) become activated. The Src-family PTKs (Lck, Fyn, Yes and Lyn) and the Syk-family PTKs (ZAP-70 and Syk) have been identified as mediators of proximal signaling in NK cells and inhibition of PTK activation has been demonstrated to block ADCC (Einspahr et al., 1991; O'Shea et al., 1992). Downstream of PTK activation, PLC- γ (Azzoni et al., 1992; Ting et al., 1992), PI3K (Kanakaraj et al., 1994), Ras, Vav (Xu and Chong, 1996), PLA2, Erk2 (Milella et al., 1997) and nuclear factor of activated T cells (Aramburu et al., 1995) have been implicated as intracellular mediators of ADCC.

Her2/Neu (c-erb-B-2) encodes a protein tyrosine kinase (Ross and Fletcher, 1998) that is expressed on approximately 20% of breast adenocarcinomas. NK cells isolated from breast cancer patients and cultured *in vitro* with IL-2 or IL-15, demonstrate high levels of ADCC against Her2/neu positive tumor cells coated with Herceptin, a mAb to Her2/Neu (Carson et al., 2001). NK cells isolated from patients administered with low dose IL-2 and then incubated *in vitro* with Her2/neu positive tumor targets coated with Herceptin also demonstrated increased ADCC when compared with NK cells isolated from patients prior to IL-2 therapy (Carson et al., 2001). An anti-MUC1-scFv-Fc-IL-2 antibody-cytokine fusion protein has also been developed that binds MUC-1 on tumor cells and induces proliferation of pre-activated lymphocytes and activation of NK cells via the IL-2 moiety (Heuser et al., 2003). *In vitro*, NK cells activated by the fusion protein were demonstrated to lyse MUC-1 positive tumors (Heuser et al., 2003). Recently, researchers at Chiron Corp. (Emeryville, CA, USA)

demonstrated that patients with advanced-stage non-Hodgkin lymphoma who were treated with the anti-CD20 mAb rituximab and rIL-2 developed increased numbers of NK cells that could recognize and eliminate antibody-coated cancer cells (O'Hanlon, 2004). Tumors were completely eliminated in some patients and decreased 25-50% in size for others. Collectively, these studies illustrate the importance of NK cell control of tumor growth mediated by ADCC.

1.2.3 Perforin (pfp)/granzyme (grz) exocytosis

Granule exocytosis is the major pathway used by NK cells to eliminate cancerous and virus-infected host cells (Figure 1.3). Upon activation, NK cells release cytoplasmic granules that contain a number of lytic proteins including pfp and a family of structurally related serine proteases (grz or granule enzymes). The grz are processed from inactive pro-enzymes into active enzymes either on route to, or in, the granules by cathepsin C (also known as dipeptidyl peptidase I) (Smyth et al., 1995), but remain inactive due to the acidic pH within the granules. Once released and transferred into the target cell cytosol (which has a relatively neutral pH of approximately 7), the grzs gain maximal activity and can activate apoptotic cysteine proteases (caspases) or exert other functions leading to target cell death. In addition to pfp and grzs, NK cell granules also contain cathepsin B, a lysosomal protease that upon degranulation is sequestered on the effector cell membrane and may inactivate pfp molecules that diffuse back upon the effector cell surface (Balaji et al., 2002).

The grzs are a family of serine proteases expressed by NK cells and cytotoxic T lymphocytes (CTLs). Many of the grzs have been shown to induce apoptosis, although some serve additional functions and are incapable of inducing a death signal (Brunner et al., 1990; Irmeler et al., 1995; Sower et al., 1996; Suidan et al., 1994; Suidan et al., 1996). Five grzs have been described in humans (grzA, B, H, K and M) and ten in mice (grzA-G, K, M and N) (Lieberman, 2003). GrzB, the most powerful pro-apoptotic member of the grz family, is an aspace and cleaves after specific aspartic acid residues such as those found in several procaspases and downstream caspase substrates (Darmon et al., 1995; Poe et al., 1991). GrzA

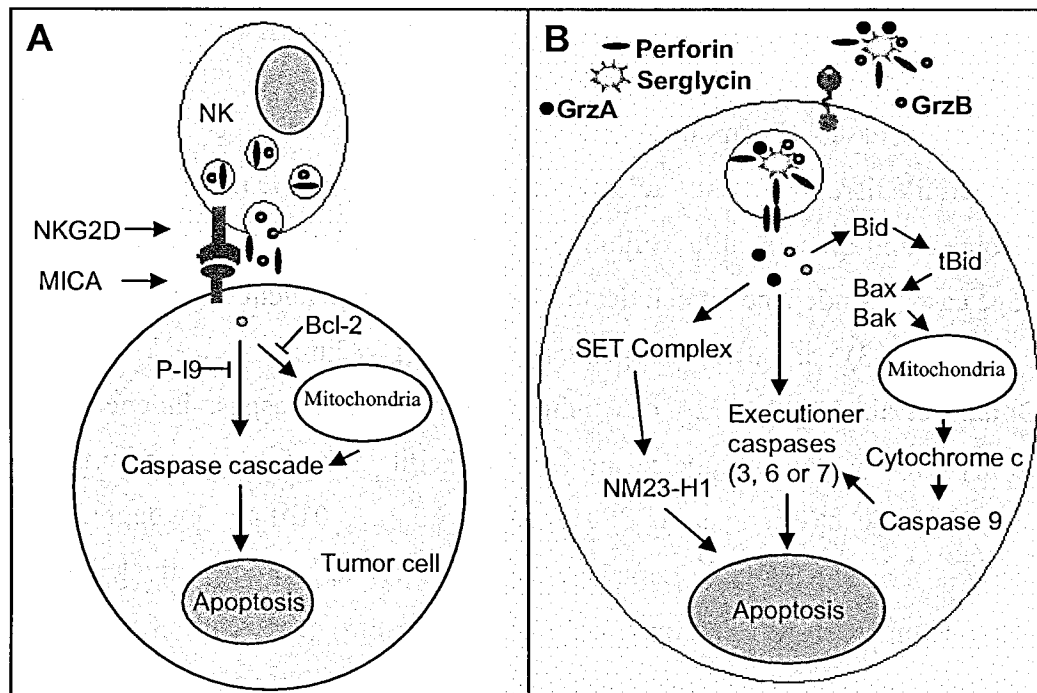


Figure 1.3. Granule exocytosis pathway. (A) Upon recognition of activating ligands such as NKG2D, NK cells can secrete granule contents to induce apoptosis by both caspase-dependent and caspase-independent mechanisms. Cellular proteins such as the serine protease inhibitor P-19 and Bcl-2 can inhibit some of the mechanisms utilized by grz. (B) Granule contents are thought to exist in a macromolecular serglycin complex that is taken up into the target cell by receptor-mediated endocytosis. The grz are then liberated from endosomes via membrane pores produced by pfp. Once grzA has entered the cytoplasm, it degrades the SET complex, which rapidly translocates to the nucleus, where NM23-H1 nicks chromosomal DNA. GrzB, on the other hand, cleaves BH3-interacting domain death agonist (Bid), which translocates to the mitochondria and stimulates the release of mitochondrial contents. Cytochrome c released from the mitochondria binds to the apoptotic protease-activating factor 1 (APAF-1) and caspase 9, forming the apoptosome, which proceeds to activate the executioner caspases, 3, 6 and 7. GrzB can also directly cleave the executioner caspases and other cellular substrates to induce apoptosis. Abbreviations: Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-associated X protein.

and K are tryptases and are predicted to cleave after arginine and lysine residues (Sattar et al., 2003; Simon and Kramer, 1994), grzH is a chymase and cleaves after phenylalanine and tyrosine residues (Edwards et al., 1999) and grzM is a metase that can cleave after methionine, leucine and norleucine residues (Smyth et al., 1992).

GrzA and B are the most abundant grz in mice and humans. GrzB induces cell death in a caspase-dependent manner by directly cleaving and activating executioner caspases (Darmon et al., 1995) and by cleaving Bid to induce mitochondrial disruption and release of pro-apoptotic mediators such as cytochrome c and Smac/Diablo (Sutton et al., 2000). GrzB has also been reported to induce caspase-independent cell death in both a Bid-dependent (Li et al., 2001; Lieberman, 2003) and Bid-independent manner (Alimonti et al., 2001; MacDonald et al., 1999). GrzA can induce apoptosis in a caspase-independent manner by directly cleaving nuclear proteins to facilitate the formation of single-stranded DNA nicks (Zhang et al., 2001a; Zhang et al., 2001b) and by degrading the newly described SET complex, allowing NM23-H1 to become activated and nick chromosomal DNA (Beresford et al., 1999; Beresford et al., 2001; Fan et al., 2003a; Fan et al., 2002; Fan et al., 2003b; Sarin et al., 1997; Trapani et al., 1998).

Although it is known that pfp and the grzs can act synergistically to induce target cell apoptosis, exactly how they achieve this has been a topic of great debate. In the early 1980s, purified pfp was thought to form polyperforin pores in target cell membranes, inducing osmotic instability and lysis of the target cell (Podack and Konigsberg, 1984). In the 1990s, discovery of the grzs and their ability to induce cell death co-operatively with pfp resulted in a modification of the original model. Grzs were thought to gain entry into the target cell by passive diffusion through pfp pores. Further research suggested that this model was unlikely for several reasons. Firstly, the pro-apoptotic function of pfp and grzs could not be replicated by replacing pfp with the complement membrane-attack complex or other membrane-disrupting proteins, which form structurally similar pores (Browne et al., 1999; Podack et al., 1988). Secondly, researchers have demonstrated that pfp and grzs are sequestered to the target cell surface as a

single macromolecular complex associated with the proteoglycan serglycin (Metkar et al., 2002). Moreover, cytosolic delivery of grzs was demonstrated to occur independently of plasma membrane pore formation (Metkar et al., 2002). Thirdly, several groups have demonstrated that grz entry into the target cell occurs via receptor-mediated endocytosis and does not require pfp (Froelich et al., 1996; Pinkoski et al., 1998; Shi et al., 1997; Trapani et al., 1998). Recently, the mannose 6-phosphate receptor (MPR) has been demonstrated to act as a receptor for grzB uptake when it is overexpressed on the cell surface (Motyka et al., 2000). However, the physiological relevance of this interaction has been questioned given that only low levels of MPR are normally found on the cell surface (Trapani and Smyth, 2002; Trapani et al., 2003).

1.2.4 Death receptor (DR) pathway

The TNF superfamily of ligands and receptors modulate innate and adaptive immune responses by inducing cellular activation, proliferation, survival, differentiation, migration and apoptosis (Smith et al., 1994; Van Parijs and Abbas, 1996) (Figure 1.4). Within this family, TRAIL/Apo2L, FasL, TNF and LT- α have been characterized as major mediators of apoptosis. TNF superfamily members exert apoptosis by engaging target cell DRs that contain cytoplasmic death domains (DDs). These DDs mediate interaction between the DR and adaptor proteins such as Fas-associated death domain (FADD) that can activate caspases and initiate apoptosis. TNF superfamily receptors can also activate nuclear factor-kappaB (NF- κ B), a transcription factor implicated in suppression of apoptosis, cell survival, proliferation, viral replication, inflammation, bone resorption, tumorigenesis and metastasis. Other pathways activated by the TNF superfamily include JUN N-terminal kinase (JNK), p42/p44 mitogen-activated protein kinase (MAPK) and p38 MAPK. Some TNF superfamily receptors contain truncated DDs or lack cytosolic regions that may allow them to act as 'decoys' and compete for binding with cognate ligands. DR-mediated apoptosis is discussed in greater detail in Chapter 1, Sections 1.3.2 and 1.3.4.

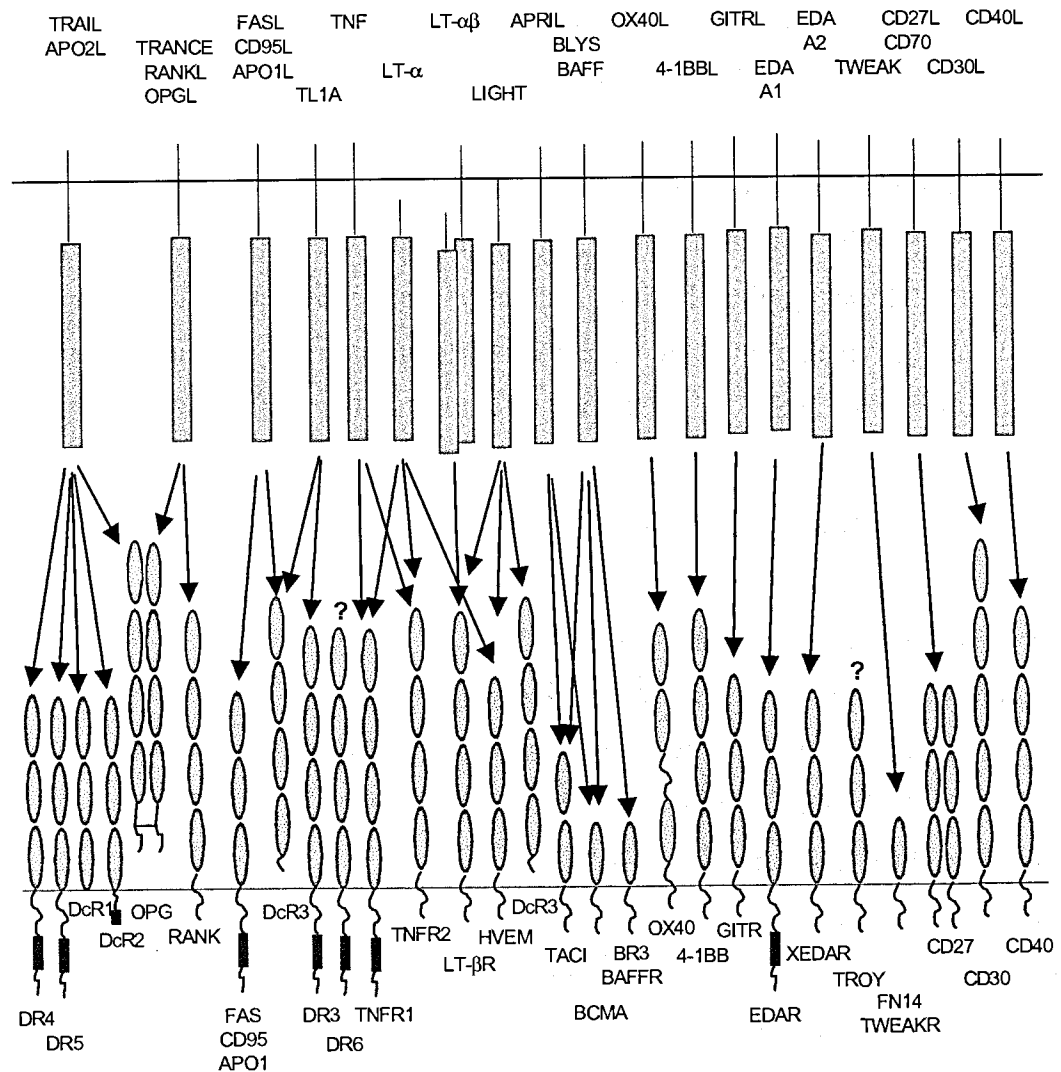


Figure 1.4. TNF superfamily receptors and ligands. Many of the TNF-superfamily of ligands (top) can bind to multiple receptors (bottom) and some ligands such as TNF and LT- α even share common receptors. Arrows indicate interactions between TNF-superfamily ligands and their corresponding receptor/s. Question marks indicate receptors for which the cognate ligand is yet to be identified. Cysteine-rich receptor domains are represented by red ovals and cytoplasmic DDs for receptors are represented by green rectangles. Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B-cell-activating receptor; BCMA, B-cell maturation antigen; Dc, Decoy; EDA, ectodermal dysplasin; EDAR, ectodysplasin-A-receptor; GITR, glucocorticoid-induced TNFR family receptor; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor- κ B; TACI, transmembrane activator and cyclophilin ligand interactor; TWEAK, TNF-like weak inducer of apoptosis; XEDAR, X-linked ectodermal dysplasia receptor. Adapted from (Ashkenazi, 2002).

1.2.5 NK cell cytokine production

Upon activation, NK cells themselves can produce a variety of cytokines [IFN- γ , GM-CSF, G-CSF, M-CSF, TNF- α , IL-5, IL-10, IL-13 and others (Peritt et al., 1998)] and chemokines [CXCL1, CCL1, CCL3, CCL4, CCL5, CCL22, CXCL8 and others (Robertson, 2002)] which can have a direct effect on tumor growth, induce inflammatory and anti-viral responses, regulate hematopoietic cell differentiation and prime immune effector cells that are crucial for subsequent adaptive immune responses (Figure 1.5). For example, IFN- γ produced by NK cells can directly stimulate the development of CTL and lead to the development of immunological memory against the tumor (Kelly et al., 2002a; Smyth and Kelly, 1999). NK cell IFN- γ can also promote the development of CD4⁺ T helper cells that subsequently aid CTL generation (Smyth et al., 2002b). Activated NK cells can also stimulate the maturation of DCs, allowing them to present antigen to CTLs in LNs (Cooper et al., 2004; Gerosa et al., 2002). In addition, cytokines produced by NK cells may also regulate B cell production of anti-tumor antibodies (Yuan et al., 1994; Yuan et al., 1992).

NK cells are important contributors to the innate defense against viral infection. NK cell IFN- γ responses have been observed during influenza virus (Monteiro et al., 1998) and MCMV infection (Orange and Biron, 1996a; Orange and Biron, 1996b; Orange et al., 1995; Ruzek et al., 1997). NK cell IFN- γ production during infection occurs as early as hours to days after the primary infection and is dependent upon virus-induced IL-12 (Monteiro et al., 1998; Orange and Biron, 1996a; Orange and Biron, 1996b). TNF produced by NK cells might also have an anti-viral function, although a number of cells can produce TNF during the early stages of viral infection (Orange and Biron, 1996b).

NK cells produce an important early source of IFN- γ in response to malaria infection (Artavanis-Tsakonas and Riley, 2002; Mohan et al., 1997). Production of IFN- γ is IL-12-dependent and partially IL-18-dependent (Artavanis-Tsakonas

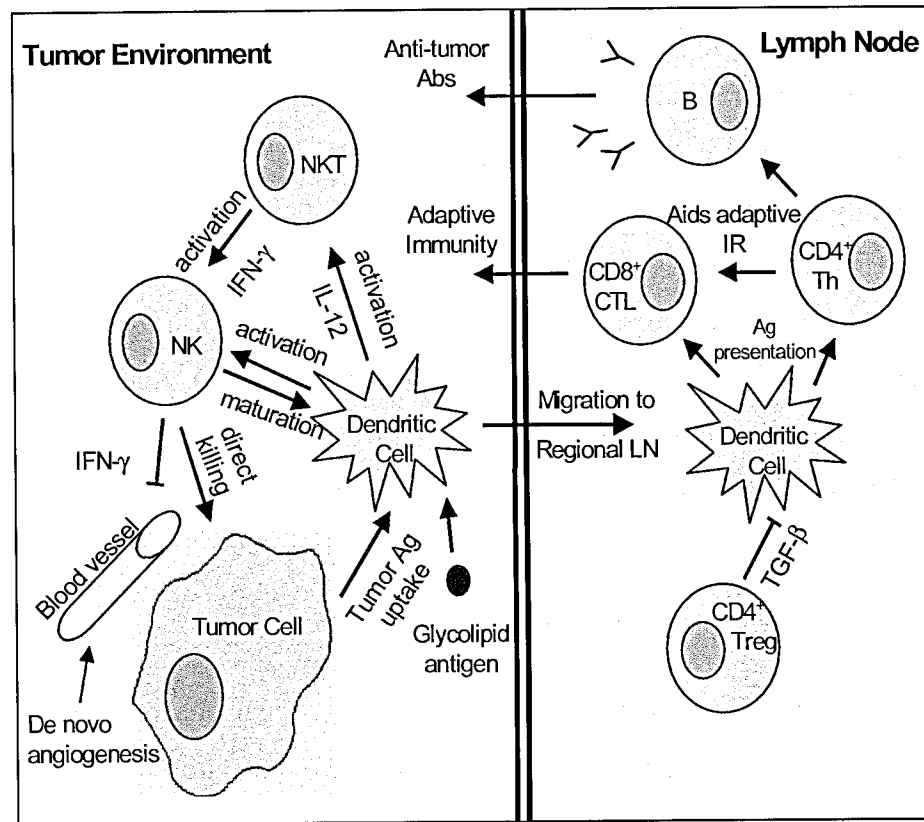


Figure 1.5. Network of the anti-tumor immune response. The immune system involves a complex network of cells that “communicate” with each-other to form an anti-tumor response. NK cells can recognize stress ligands expressed by tumor cells and mediate either direct killing or produce IFN- γ to suppress tumor cell angiogenesis. Immature DCs take up tumor antigens and then migrate to the regional LN where they present antigen to CD4⁺ and CD8⁺ T cells. CD4⁺ T cells help generate cytolytic CD8⁺ T cells that can migrate to the tumor site and kill tumor cells. CD4⁺ T cells also regulate B cell production of anti-tumor antibodies (Abs). CD25⁺CD4⁺ T regulatory cells suppress anti-tumor immunity by the secretion of immunomodulatory cytokines such as TGF- β . Stimulated NK cells aid NK cell maturation, whilst DC secreted cytokines help further activate NK cells. DCs can also present glycolipids in the context of CD1d, activating NKT cells, which in turn further activate NK cells and adaptive immunity.

and Riley, 2002) and acts synergistically with TNF to stimulate nitric oxide production (Jacobs et al., 1996) that is important for malaria parasite killing (Rockett et al., 1991). NK cell IFN- γ production has also been associated with early host resistance against infection with *Mycobacterium tuberculosis* (Junqueira-Kipnis et al., 2003; Vankayalapati et al., 2004). A recent study proposed that NK cell IFN- γ stimulates secretion of IL-15 and IL-18 by monocytes which in turn favors the expansion of CD8⁺IFN- γ ⁺ T cells that can lyse infected cells (Vankayalapati et al., 2004).

Two distinct human NK cell subsets have been identified which can be distinguished by cell surface density expression of CD56. CD56^{bright} NK cells comprise approximately 10% of human NK cells and CD56^{dim} NK cells that express high levels of Fc γ RIII (CD16) comprise approximately 90% of the population (Lanier et al., 1986). Resting CD56^{dim} NK cells demonstrate enhanced cytotoxicity against NK-sensitive targets compared with CD56^{bright} resting NK cells (Nagler et al., 1989), although both NK cell subsets exhibit similar levels of NK cell cytotoxicity after stimulation with cytokines such as IL-2 or IL-12 (Ellis and Fisher, 1989; Nagler et al., 1989; Robertson et al., 1992). CD56^{bright} NK cells are potent producers of a variety of cytokines (IFN- γ , TNF- α , TNF- β , IL-10, IL-13 and GM-CSF) in response to monocyte-derived cytokines (monokines) (Cooper et al., 2001). This suggests that CD56^{bright} and CD56^{dim} NK cells represent two functionally distinct subsets of NK cells in humans, one with relatively poor cytolytic capacity but the capability to produce large amounts of cytokines and the other which exhibits enhanced natural cytotoxicity but produce significantly less cytokines. Mouse NK cells do not express CD56, precluding comparative studies being undertaken in mice.

NK cell maturation studies have identified 3 stages of NK cell differentiation (detailed in Chapter 1, Literature Review, Section 1.2.1). The most immature (type 2) NK cells produce type 2 cytokines such as IL-13 and IL-5, intermediate (type 0) NK cells are reported to produce both IL-13 and IFN- γ and the most mature (type 1) NK cells produce IFN- γ (Loza and Perussia, 2001). Mature IFN- γ -producing NK cells were CD56^{bright} whereas immature NK cells that did not

produce IFN- γ were CD56^{dim} (Loza and Perussia, 2001), in agreement with studies illustrating that CD56^{bright} NK cells are more potent cytokine producers (Cooper et al., 2001).

1.2.6 Tumor rejection by NK cells

In the 1970s, NK cells were defined as white blood cells that could spontaneously lyse the tumor targets YAC-1 (A/Sn mouse Moloney tumor virus-induced lymphoma cell line) (Kiessling et al., 1975) and K562 (human chronic myeloid leukemia cell line) (Ortaldo et al., 1977). Subsequently, cytokine- (IL-2, IFN- α , IFN- β) activated NK cells were demonstrated to exhibit enhanced cytolytic function and to kill a broader range of tumor targets, including some tumors that were resistant to lysis by resting NK cells (Grimm et al., 1982; Henney et al., 1981; Phillips and Lanier, 1986). NK cells are now recognized as lymphocytes with the unique ability to lyse class I MHC-deficient tumor cells and their metastases (Whiteside and Herberman, 1995), a skill that enables them to kill cancerous cells that have downregulated class I MHC to evade the adaptive immune response.

The importance of NK cells in the anti-tumor response has been clearly established in a number of experimental tumor models. Many of these studies have used NK cell depletion prior to tumor transplantation to demonstrate more aggressive tumor growth in the absence of NK cells (Gruber et al., 2002; Liao et al., 1991; Smyth et al., 1999). Others have used cytokines (IL-2, IL-21, IL-12, IL-15, IL-18 and IFNs) to boost the NK cell anti-tumor response, demonstrating that enhanced NK cell function leads to more efficient elimination of tumor growth (Brady et al., 2004; Smyth et al., 2000a; Talmadge et al., 1987; Trinchieri, 1998; Wang et al., 2003; Wiltout et al., 1985). Adoptive transfer of NK cells has also clearly demonstrated the ability of NK cells to mount an anti-tumor response (Barlozzari et al., 1983; Kuppen et al., 2001; Yasumura et al., 1994).

It has previously been shown that NK cell-mediated rejection of spontaneous tumor development in a variety of murine models is largely mediated by pfp and IFN- γ (Street et al., 2001). IFN- γ produced by NK cells might play a role in the regulation of killing by DRs by downregulating the expression of intracellular FLICE inhibitory proteins (FLIPs) (Irmeler et al., 1997; Thome et al., 1997), or by upregulating expression of caspases that are essential for the execution of DR-mediated apoptosis (Griffith et al., 1998). IFN- γ produced by NK cells and NKT cells is essential for the anti-angiogenic effects of α -GalCer on tumor growth, and NK cell depletion partially inhibits angiogenesis *in vivo* (Hayakawa et al., 2002b). IL-12-mediated inhibition of tumor angiogenesis has also been demonstrated to be mediated by NK cells (Yao et al., 1999), although it has been shown that low doses of IL-12 preferentially activates NKT cells rather than NK cells (Smyth et al., 2000a; Takeda et al., 2000a).

Although the importance of NK cells in control of tumor growth has been studied extensively, establishing whether NK cells can protect against tumor initiation has been more difficult. Early on, "NK cell defective" *beige* mice were used, however these mice have a defect in cytoplasmic granule formation that is also known to affect CTLs and macrophages (Ashman and Papadimitriou, 1991; Roder, 1979). Numerous studies have been undertaken using anti-NK1.1 mAb to deplete NK cells, however this antibody deletes both NK cells and NKT cells and the NK1.1 marker is only expressed on NK cells from mice of specific backgrounds. Patients lacking NK cells have been described, however, these patients are extremely susceptible to viral infection and die at an early age (Ballas et al., 1990; Biron et al., 1989). Familial haemophagocytic lymphohistiocytosis (FHL) patients who possess pfp mutations and lack NK cell activity have also been described, but die at a young age unless rescued by a BM transplant (BMT) (Stepp et al., 1999).

Key studies performed by Smyth and colleagues demonstrated that mice depleted of NK cells were more susceptible to spontaneous tumor growth initiated by methylcholanthrene (MCA) (Smyth et al., 2001b; Smyth et al., 2000b). NK cells are also capable of rejecting spontaneous β 2 μ -deficient B cell lymphomas in a

pfp-dependent manner. Recently, a spontaneous resistance/complete resistance (SR/CR) mouse was identified by Cui and colleagues (Cui et al., 2003). The original founder mouse was identified due to a failure to develop ascites after repeated injection with S180 sarcoma cells. Breeding experiments revealed the trait was a dominant gain of function mutation and further analysis revealed an age-dependent spontaneous regression of advanced cancers. Young mice (6 weeks of age) displayed a CR phenotype, 50% of mice 12 weeks of age displayed a CR phenotype and 50% displayed a SR phenotype and the majority of 22 week old mice displayed a SR phenotype. Resistance in these mice was associated with an infiltration of innate leukocytes including NK cells, neutrophils and macrophages into the peritoneum. This model suggests that a host resistance mechanism against cancer may exist, that the mechanism may involve NK cells and that resistance may decline with age.

NK cells isolated from cancer patients exhibit impaired NK cell cytotoxicity, proliferation, proliferation and response to IFNs and patients with higher levels of NK cell activity post treatment are reported to remain cancer-free longer than patients with low NK cell activity (Whiteside and Herberman, 1994; Whiteside et al., 1998). The importance of NK cells in control of tumor development is also illustrated in patients with XLP syndrome. These patients have defective SAP that prevents NK cell activation through CD244 (2B4) (Benoit et al., 2000; Nakajima et al., 2000; Nichols et al., 1998; Sayos et al., 1998). As a consequence, SHP-1 is stably associated with 2B4 in patient NK cells, causing 2B4 to act as an inhibitory rather than an activating receptor (Parolini et al., 2000). XLP patients are highly susceptible to Epstein Barr virus (EBV) infection and develop B-cell lymphomas suggesting that NK cells may control EBV infection in these patients (Wu and Lanier, 2003).

BMT is commonly used to treat leukemia patients at less advanced stages of disease, however, tumor relapse remains a serious issue. Recently, Velardi and colleagues demonstrated that BMT using haploidentical, but KIR mismatched donors induced NK cell alloreactions that could mediate a GVL effect and

prevent tumor relapse in patients (Figure 1.6) (Ruggeri et al., 2002). Moreover, higher rates of engraftment were obtained and graft-versus-host disease was prevented, most likely via elimination of host antigen-presenting cells (APCs). Another study suggested that KIR mismatch provides no advantage in mediation of GVL effects (Davies et al., 2002), however these differences might be explained by numbers of stem cells transplanted, post-grafting immune suppression and T cell depletion. NK cells may also play a role in IFN- α -mediated treatment of chronic myelogenous leukaemia (CML) as NK cells isolated from patients treated with IFN- α exhibit increased cytotoxicity against autologous CML blasts (de Castro et al., 2003; Pawelec et al., 1995).

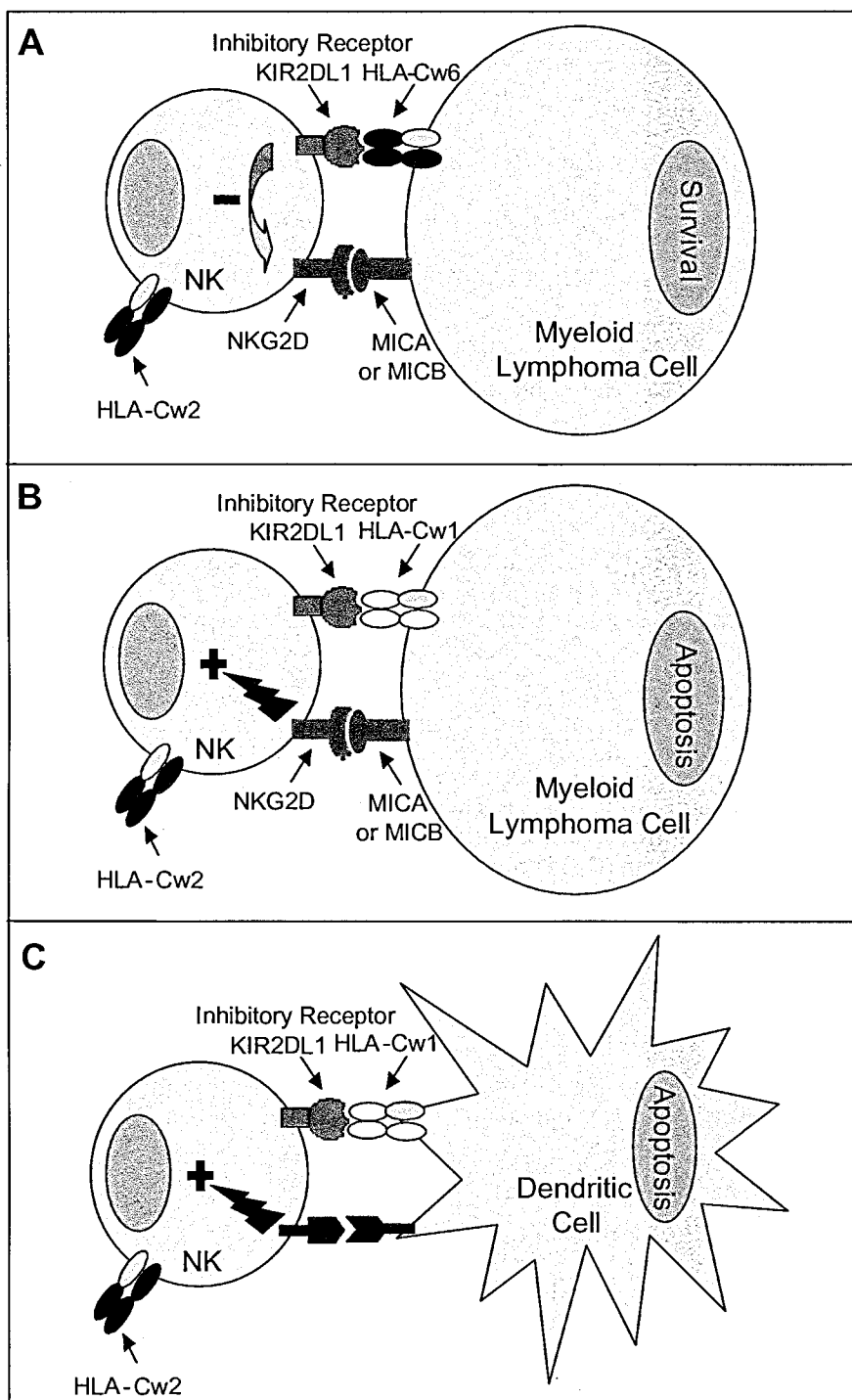
In some cases, NK cells may co-operate with other immune effector cells to eliminate tumor. For example, both CD8⁺ T cells and NK cells are required to protect mice from *in vivo* growth of B16-CD80 transfectants (Wu et al., 1995). NK cells also facilitate an adaptive anti-tumor immune response by producing IFN- γ and other cytokines to activate DCs, T cells and B cells (Smyth et al., 2002b) (detailed in Chapter 1, Literature Review, Section 1.2.5).

1.2.6.1 α -Galactosylceramide (α -GalCer) and bystander NK cell activation

(2S,3S,4R)-1-O-(α -D-galactopyranosyl)-2-(N-hexacosanoyl-amino)-1,3,4-octadecanetriol (α -galactosylceramide or α -GalCer) is a CD1d-binding glycolipid originally identified in an extract from the marine sponge *Agelas mauritanus* (Morita et al., 1995) (Figure 1.7). Since these early studies, α -GalCer has been shown to act as a specific CD1d restricted ligand for V α 14 invariant-NKT (iNKT) cells, powerfully stimulating them to produce high levels of IFN- γ and IL-4 (Kawano et al., 1997).

The anti-tumor activity of α -GalCer has been demonstrated for a wide variety of tumors of different origins including; breast, lung, kidney and colon carcinomas, melanoma as well as hematopoietic malignancies (Morita et al., 1995; Kawamura

Figure 1.6. NK cells in GVL. (A) When donor and recipient are haploidentical, inhibitory KIR receptors can bind to HLA molecules of the same supertype, thereby sending an inhibitory signal to donor NK cells upon binding to leukemia targets. In this example, KIR2DL1 is inhibited by group 2 HLA-C alleles (HLA-Cw2,4,5 and 6) and the HLA-Cw2 expressing NK cell is prevented from killing the HLA-Cw6 expressing leukemia cell. (B) When the donor and recipient are KIR mismatched (in this example the recipient expresses the group 1 HLA-C allele HLA-Cw1), the HLA-Cw2 expressing KIR2DL1⁺ NK cells are no longer inhibited and kill the leukemia cell. (C) NK cells also reduce graft-versus-host disease by eliminating KIR mismatched host antigen-presenting cells.



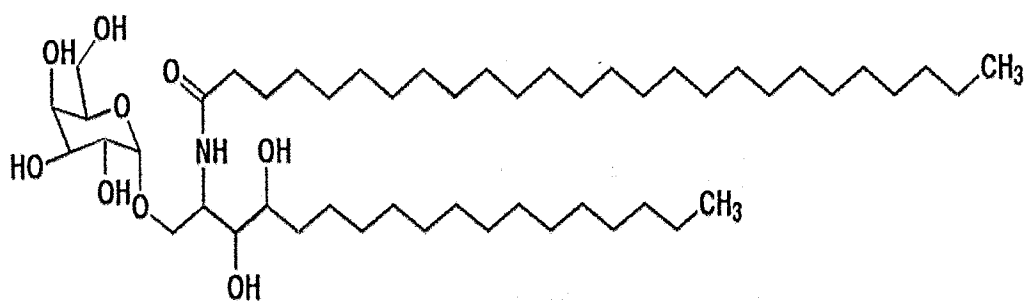


Figure 1.7. Structure of α -GalCer. The α -anomeric conformation of the sugar moiety is critical for optimum iNKT cell activation. The length of the long chain sphingosine base (C₁₄) and fatty acyl chain (C₂₆) is considered suitable for binding to CD1d via hydrophobic interactions

et al., 1998; Kobayashi et al., 1995; Nakagawa et al., 1998; Smyth et al., 2001a; Yamaguchi et al., 1996). α -GalCer has also been shown to inhibit tumor initiation in several spontaneous tumor models including; MCA-treated mice, p53-deficient mice and Her-2/neu transgenic mice (Hayakawa et al., 2004).

Cross-talk between α -GalCer-stimulated iNKT and other immune cells is crucial for amplification of the α -GalCer response. Although stimulation of iNKT with α -GalCer results in rapid production of Th1 and Th2 cytokines, this is followed by a massive IFN- γ -dependent proliferation and activation of NK cells (Bendelac et al., 1997; Carnaud et al., 1999; Eberl and MacDonald, 2000; Hong et al., 1999; Kawano et al., 1997; Singh et al., 1999) and NK cell IFN- γ production (Hayakawa et al., 2001). Moreover, the anti-metastatic effects of α -GalCer are impaired in IFN- $\gamma^{-/-}$ and NK cell-depleted mice (Hayakawa et al., 2001; Smyth et al., 2002a) and α -GalCer-induced *in vitro* cytotoxicity is ameliorated in hepatic MNC isolated from IFN- $\gamma^{-/-}$ and NK cell-depleted mice (Hayakawa et al., 2001). Furthermore, NKT cell IFN- γ production alone is not sufficient to mediate a significant anti-tumor response (Smyth et al., 2002a). In addition to NK cells, bystander activation of conventional T and B cells and DCs also occurs (Burdin et al., 1999; Carnaud et al., 1999; Cui et al., 1999; Eberl and MacDonald, 2000; Kitamura et al., 2000; Nishimura et al., 2000; Singh et al., 1999).

1.3 Death receptors (DRs)

To date, eight members of the DR family have been identified: TNFR1 (CD120a), CD95 (Fas/APO1), DR3 (APO-3, LARD, TRAMP, WSL1), TRAIL-R1 (APO-2, DR4), TRAIL-R2 (DR5, KILLER, TRICK2), DR6, p75-NGFR and EDAR. Some TNF superfamily receptors contain truncated DDs or lack cytosolic regions that may allow them to act as 'decoys' and compete for binding with cognate ligands.

1.3.1 Structure

The DRs are type I transmembrane proteins. Their extracellular domains are characterized by 3 or 4 cysteine-rich domains (CRDs) that are pseudo-repeats that commonly contain 6 cysteine residues engaged in the formation of 3 disulphide bonds (Bodmer et al., 2002). The amino terminal extracellular region has a high degree of homology (~65% protein sequence identity). Receptor structure is stabilized by a 'twisted ladder' of disulphide bridges (Bodmer et al., 2000b). Soluble forms of the DRs can be generated by proteolytic processing (eg. TNFR1) (Gruss and Dower, 1995) or by alternative splicing of the exon encoding the transmembrane domain (eg. Fas) (Smith et al., 1994).

The DR ligands are type II transmembrane proteins that share a conserved (~30% protein sequence identity) C-terminal TNF homology domain (THD). The THD binds to the CRDs of the DRs allowing transmission of the death signal. With the exception of LT- α that lacks a transmembrane domain and is secreted as a homotrimer, the death ligands (including heterotrimeric LT α 1 β 2) can be cleaved from the membrane by metalloproteinases to generate soluble forms that are released into the extracellular space (Black et al., 1997; Chen et al., 2001b; Lum et al., 1999; Powell et al., 1999; Schneider et al., 1999). Whilst the function of some ligands is dependent upon solubilization, the function of others such as FasL is downregulated upon cleavage (Tanaka et al., 1998).

The crystal structure of human LT- α bound to its receptor, human TNFR1, was published by Banner and colleagues in 1993 (Banner et al., 1993). Three receptors were identified to assemble with 3 ligands forming a hexameric complex. A similar crystal structure was also identified for TRAIL bound to its receptor TRAIL-R2 (Cha et al., 2000; Hymowitz et al., 1999; Mongkolsapaya et al., 1999) where 3 receptors were established to bind 3 ligands, suggesting that the 3:3 stoichiometry is likely the basis of a signaling unit for the DRs and their corresponding ligands.

1.3.2 Signaling

FasL or TRAIL induce target cell apoptosis by engaging and activating the DRs Fas and DR4/DR5 respectively (Figure 1.8). Upon activation, the receptors trimerize which allows clustering of the DDs located within their cytoplasmic regions and formation of the death-inducing signaling complex (DISC) (Kischkel et al., 1995; Kischkel et al., 2000). The adaptor protein FADD is recruited to the clustered receptor DDs by homotypic DD interactions and recruits and activates caspase-8 and/or 10 (Bodmer et al., 2000a; Kischkel et al., 1995; Kischkel et al., 2000; Kischkel et al., 2001; Sprick et al., 2000; Wang et al., 2001b). This leads to proteolytic activation of executioner caspases such as caspase-3 and ultimately to death of the target cell. TNF binding to TNFR1 induces target cell apoptosis in a slightly different manner. Rather than directly recruiting FADD to the receptor, the adaptor protein TNFR-associated death domain (TRADD) is initially recruited which secondarily recruits FADD (Hsu et al., 1996). The cell death pathway mediated by DR/ligand interaction has been termed the 'cell-extrinsic' pathway (Ashkenazi, 2002).

The DRs can also activate the 'cell-intrinsic' pathway (Ashkenazi, 2002) to induce target cell death via caspase-8-mediated cleavage of Bid (Gross et al., 1999; Li et al., 1998; Luo et al., 1998; Yin et al., 1999) (Figure 1.8). Truncated Bid (tBid) translocates to the mitochondria where it interacts with Bax and Bak stimulating the release of cytochrome c and Smac/Diablo (Adams and Cory, 1998; Eskes et al., 2000; Wei et al., 2000). Cytochrome c liberated from the mitochondria then binds to the adaptor protein APAF1, forming the apoptosome and activating caspase-9 which in turn activates executioner caspases leading to target cell death. Smac/Diablo promotes apoptosis by blocking inhibitor of apoptosis (IAP) proteins (Du et al., 2000; Verhagen et al., 2000). Cross-talk between the 'cell-extrinsic' and 'cell-intrinsic' pathways following DR activation may be important for amplification of the death signal in some cells.

Signaling pathways downstream of the DRs can be regulated in a number of ways. Cellular FLICE-inhibitory proteins (c-FLIPs) are homologous to caspase-8

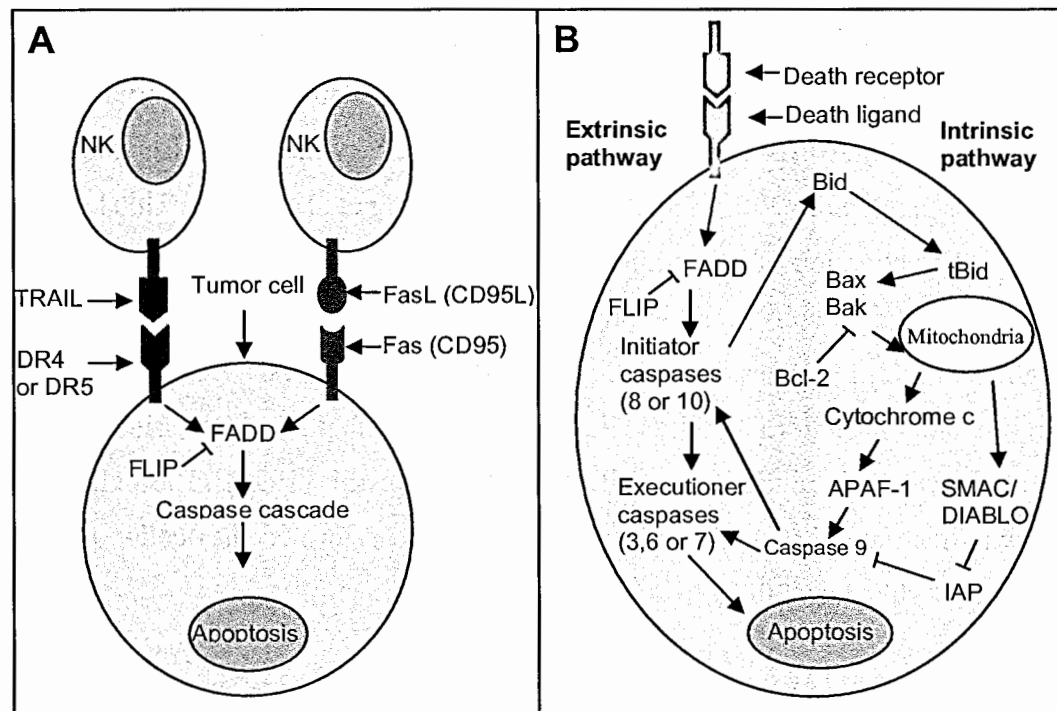


Figure 1. 8. DR pathway. (A) NK cells which express TNF superfamily ligands such as FasL or TRAIL can induce target cell apoptosis by engaging and activating DRs such as Fas or DR4/DR5. DRs within the cytoplasmic region of these receptors then engage adaptor proteins such as FADD that can recruit and activate caspases, ultimately leading to death of the target cell. FLIPs can interfere with the generation of active caspase-8. (B) DRs can activate both 'cell-intrinsic' and 'cell-extrinsic' pathways to induce target cell death. The 'cell-extrinsic' pathway triggers apoptosis via activation of initiator caspases (8 and 10) followed by activation of executioner caspases (3, 6 or 7). DRs can also activate the 'cell-intrinsic' pathway via caspase-8-mediated cleavage of Bid. tBid translocates to the mitochondria where it interacts with Bax and Bak stimulating the release of cytochrome c and Smac/ Diablo. Cytochrome c liberated from the mitochondria then binds to the adaptor protein APAF1, forming the apoptosome and activating caspase-9 which in turn activates executioner caspases leading to target cell death. Smac/Diablo promotes apoptosis by blocking IAP proteins. Crosstalk between the 'cell-extrinsic' and 'cell-intrinsic' pathways following DR activation may be important for amplification of the death signal in some cells.

and 10 (Tschopp et al., 1998) and can prevent recruitment of caspase-8 or 10 to the DISC and hence the activation of these caspases. Viral FLICE-inhibitory proteins (v-FLIPs) encoded by several members of the herpes virus family (Bertin et al., 1997; Hu et al., 1997; Searles et al., 1999; Thome et al., 1997) similarly protect against cell death induced by activation of members of the TNF receptor family. The poxvirus encoded protein crmA which is homologous to a serine protease inhibitor (serpin) also inhibits DR-mediated apoptosis by blocking caspases (Cassens et al., 2003; Komiyama et al., 1996). Chaperones have been shown to modulate DR-mediated apoptosis, for example apoptosis induced by TNF can be blocked by the heat-shock protein Hsp70 (Jaattela et al., 1998). Protein kinase C (PKC) has been reported to inhibit the recruitment of DD-containing proteins that are important for DR-mediated signaling (Harper et al., 2003). In addition, MAPK (Frese et al., 2003) and Akt (Thakkar et al., 2001) activity have been demonstrated to affect TRAIL sensitivity. Expression of Bcl-2/X_L and Bax/Bak or lack thereof is particularly important for regulation of DR signaling via the 'cell-intrinsic' pathway (Burns and El-Deiry, 2001; LeBlanc et al., 2002; Scaffidi et al., 1998; Wei et al., 2001; Wen et al., 2000).

In addition to apoptosis, the DRs also regulate a number of other cellular processes including proliferation, viral replication, suppression of apoptosis, cell survival, inflammation, bone resorption and differentiation via activation of pathways involving NF κ B, JNK, p42/p44 MAPK and p38 MAPK (Degli-Esposti et al., 1997a; Hu et al., 1999; Lin et al., 2000; Natoli et al., 1998; Schneider et al., 1997).

1.3.3 Cellular expression

The TNF superfamily of ligands are expressed by a wide variety of cells within the immune system, including monocytes, NK, T, B and DCs (Table 1.2). TNF superfamily receptors are expressed by a variety of cell types including those of the immune system and others including lung, colon and epithelial cells (Table 1.2).

Ligand	Cellular expression	Receptor	Cellular expression
TRAIL	NK, T and DCs	DR4, DR5, DcR1, DcR2, OPG	Most normal and transformed cells Most normal and transformed cells
FasL	Activated splenocytes, thymocytes, immune privileged sites such as the eye and testis	Fas	Most normal and transformed cells
TNF	Macrophages, NK, T and B cells	TNFR1 TNFR2	Most normal and transformed cells Immune cells and epithelial cells
LT- α	NK, T and B cells	TNFR1, TNFR2	See above
LT- β	NK, T, B, DCs, macrophages	LT- β R	NK, CD4+ and CD8+ T cells
TWEAK	Monocytes	FN14	Fibroblasts and endothelial cells
LIGHT*	T cells, DCs, monocytes and granulocytes	HVEM LT- β R	T cells Non-lymphoid haematopoietic cells and stromal cells
		DcR3	Lung and colon cells
CD27L	NK, T and B cells	CD27	CD4+ and CD8+ T cells
CD30L	T cells and monocytes	CD30	Reed-Sternberg cells
CD40L	T and B cells	CD40	Reed-Sternberg cells
OX40L	T and B cells	OX40	T cells
4-1BBL	B cells, DCs and macrophages	4-1BB	Activated T cells, monocytes and NK cells
BAFF	T cells, DCs, monocytes and macrophages	BAFFR TACI BCMA	B cells, PBLs, LNs, spleen and resting T cells B cells, PBLs, activated T cells, thymus, spleen, intestine B cells, PBLs, thymus, spleen, LNs, liver and adrenals
APRIL	Macrophages, lymphoid cells, tumor cells	BCMA TACI	See above See above
TL1A*	Endothelial cells	DR3, DcR3	Activated T cells
GITRL	Not determined	GITR	CD4+CD25+ T cells
RANKL	Activated T cells and osteoblasts	RANK OPG	Osteoclasts, osteoblasts and activated T cells Osteoclast precursors, endothelial cells
EDA1	Skin	EDAR	Ectodermal derivative
EDA2	Skin	XEDAR	Ectodermal derivative
Not determined		DR6	Resting T cells
Not determined		RELT	Lymphoid tissues
Not determined		TROY	Embryo skin, epithelium, hair follicles and brain

Table 1.2. Cellular expression of TNF superfamily ligands and their cognate receptors. *
 Binds to DcR3. Adapted from (Aggarwal, 2003).

Although TRAIL mRNA had been identified in a number of tissues and in a variety of cell types (Wiley et al., 1995) less is known of TRAIL expression at the protein level. Initially, TRAIL was shown to be constitutively expressed by human CD4⁺ T cell clones and to mediate cytotoxicity against TRAIL-sensitive tumor cells *in vitro* (Kayagaki et al., 1999b; Thomas and Hersey, 1998). Later studies reported a lack of TRAIL expression by freshly isolated splenic lymphocytes, thymocytes and cells isolated from LNs (Kayagaki et al., 1999c). In the same study, stimulation of T cells using anti-CD3, PHA, CD40L, lipopolysaccharide and PMA plus ionomycin did not induce TRAIL expression, although TRAIL expression could be induced on splenic NK cells after stimulation with IL-2 or IL-15. Peripheral blood T cells stimulated with type I IFNs plus anti-CD3 were also identified to express TRAIL (Kayagaki et al., 1999a), as were IFN-stimulated monocytes and DCs (Fanger et al., 1999; Griffith et al., 1999b).

Key studies performed in our laboratory have identified TRAIL to be constitutively expressed on a proportion of mouse liver NK cells (Takeda et al., 2001). IFN- γ ^{-/-} and IFN- γ R^{-/-} mice lack TRAIL expression, although expression can be induced by administration of rIFN- γ . This study suggests that IFN- γ regulates constitutive TRAIL expression *in vivo* (Takeda et al., 2001). More recently, constitutive TRAIL expression was identified on numerous human and mouse ocular tissues, including corneal endothelium and epithelium, the iris, ciliary epithelium and neurosensory retina (Lee et al., 2002).

FasL is expressed on activated T cells (Suda et al., 1993), NK cells (Oshimi et al., 1996) and on tumor cells (Hahne et al., 1996; O'Connell et al., 1996; Strand et al., 1996; Tanaka et al., 1996). FasL is also expressed in several non-lymphoid cells and tissues including macrophages, DCs, epithelial cells and others (Bellgrau et al., 1995; French et al., 1997; Lu et al., 1997; Suss and Shortman, 1996). In addition, FasL expression has been reported in immune privileged sites including the CNS, testis, ovary and placenta (D'Alessio et al., 2001; French et al., 1996; Hunt et al., 1997) and like TRAIL is expressed on the corneal epithelium and retina of the eye (Ferguson and Griffith, 1997; Griffith et al., 1995). FasL

expression in immune privileged sites is important to protect these tissues from cellular immune-mediated damage (Griffith et al., 1995; Griffith et al., 1996).

TNF is produced by activated macrophages and T cells, NK cells and B cells (Carswell et al., 1975; Ware et al., 1992). Expression of TNF is transient (lasting only a few hours), dependent on TNF gene transcription and is rapidly induced by stimuli that can characteristically activate T cells (Smyth and Johnstone, 2000). TNF produced by activated macrophages and CTLs can exist as both membrane-associated and proteolytically released soluble forms that are similarly capable of killing TNF-sensitive targets (Decker et al., 1987; Monastra et al., 1996; Ratner and Clark, 1993). Two major subsets of TNF-producing activated CD8⁺ T cells exist (Hamann et al., 1997). The first 'memory-type' population express elevated levels of Fas and secrete TNF, IFN- γ and IL-4. The second, 'CTL-type' population, express CD45RA, lack co-stimulatory molecule expression, secrete both TNF and IFN- γ and are highly cytolytic (produce pfp and grzB).

1.3.4 DR-mediated apoptosis and anti-tumor activity

Cell death (apoptosis) can be activated by a number of stimuli including ultra-violet or γ -irradiation, chemotherapeutic drugs, growth factor withdrawal and by activation of the DRs. The DRs are part of the TNF superfamily and contain an intracellular DD that is essential for transduction of an apoptotic signal. Although the DRs initiate apoptosis via the 'cell-extrinsic' pathway, the apoptotic signal can be amplified by cross-talk with the 'cell-intrinsic' pathway (detailed in Chapter 1, Literature Review, Section 1.3.2). The DRs have been implicated in the control of tumor growth, homeostasis of the immune system and some appear to play a role in control of autoimmune disease. Many of these functions can be attributed to DR-mediated induction of apoptosis.

An outstanding question posed by immunologists is why expression of the TNF superfamily receptors does not always confer susceptibility to ligand-mediated stimulation. For example, TNFR1 is expressed by all cells yet the growth of only

a portion of these cells is modulated by TNF (Sugarman et al., 1985). Furthermore, TNFR1 is capable of functioning in all cells as NF κ B activation is uniformly mediated by TNF. Some reports suggest that the presence of decoy receptors can inhibit ligand stimulation (Marsters et al., 1997; Mongkolsapaya et al., 1998; Pan et al., 1998b; Sheridan et al., 1997), although decoy receptor expression does not always correlate with an inhibition of signaling (Clodi et al., 2000; Griffith et al., 1998; Griffith et al., 1999a; Zhang et al., 1999). The presence or absence of signaling molecules downstream of the DRs, such as the c-FLIPs and IAPs might also play a role in controlling susceptibility to DR-mediated apoptosis. Indeed, studies have shown a correlation between the levels of these proteins and cell death (Griffith et al., 1998; Guo et al., 2002; Kim et al., 2000). Activation of c-Myc has also been shown to sensitize cells to induction of apoptosis by TNFR1, Fas and TRAIL receptors (Hueber et al., 1997; Klefstrom et al., 1994; Lutz et al., 1998).

1.3.4.1 TRAIL

TRAIL is a type II transmembrane protein and member of the TNF-superfamily of ligands. In humans, TRAIL binds to two death-inducing receptors, DR4/TRAIL-R1 (Pan et al., 1997b) and DR5/TRAIL-R2/KILLER (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997; Wu et al., 1997). TRAIL has also been shown to bind 3 decoy receptors, DcR1/TRID/TRAIL-R3 (Degli-Esposti et al., 1997b; Pan et al., 1997a; Sheridan et al., 1997), DcR2/TRAIL-R4 (Degli-Esposti et al., 1997a; Marsters et al., 1997) and a soluble receptor OPG that was originally identified as a receptor for OPGL/RANKL (Emery et al., 1998). DcR1 completely lacks a cytosolic region and is linked to the plasma membrane by a glycosphospholipid moiety, DcR2 has a truncated cytoplasmic DD and cannot exert apoptosis. In the mouse, only one death-inducing receptor has been described that shares homology to human TRAIL-R2 (mouse DR5) (Wu et al., 1999a). Two murine decoy receptors have also been described for TRAIL (mDcTrailr1 and mDcTrailr2) (Schneider et al., 2003).

A number of studies using rTRAIL have demonstrated selective ability of TRAIL to induce apoptosis in transformed and virus-infected cells, but not in most normal cells, making it particularly attractive as a potential cancer therapeutic (Ashkenazi et al., 1999; Walczak et al., 1999) (detailed in Chapter 1, Literature Review, Section 1.4). However, little is known of the true physiological role of natural TRAIL or the expression of TRAIL at the protein level. It was not until the development of three key reagents, the anti-mouse (m)TRAIL mAb (N2B2) (Kayagaki et al., 1999c), soluble TRAIL receptor (DR5) (Song et al., 2000) and the TRAIL-deficient mice described in this study, that these questions have begun to be addressed.

Although a number of studies have shown that human T cell clones and cytokine stimulated NK cells can kill tumor cells in a TRAIL-mediated fashion (Kayagaki et al., 1999b; Kayagaki et al., 1999c; Thomas and Hersey, 1998), the anti-tumor activity of TRAIL *in vivo* has been less well studied. Key studies in our laboratory have shown that freshly isolated (unstimulated) TRAIL-expressing liver NK cells can mediate TRAIL-dependent spontaneous cytotoxicity against tumor targets *in vitro* (Takeda et al., 2001). TRAIL-expressing liver NK cells were also shown to play a role in suppression of TRAIL-sensitive L929, LB27.4 and Renca liver metastasis *in vivo*. This key study was the first to identify a role for natural TRAIL in tumor immune surveillance.

1.3.4.2 TNF

TNF was originally discovered as a serum factor that could kill cancer cells in mice (Carswell et al., 1975; Helson et al., 1975; O'Malley et al., 1962). TNF binds as a homotrimer to two receptors, TNFR1 (p55) and TNFR2 (p75) (Lewis et al., 1991). TNF- α is a type II transmembrane protein that has also been reported to exist in a soluble (proteolytically released) form and is produced by macrophages, NK, T and B cells (Carswell et al., 1975; Ware et al., 1992). The apparent non-specific toxicity of TNF might be due to the expression of TNFR1 by all cells.

TNF^{-/-} and TNFR1^{-/-} mice have been reported to lack B-cell follicles and germinal center (GC) formation (Korner et al., 1997a; Pasparakis et al., 1996) which has been attributed to defects in induction of lymphocyte homing chemokines (Ngo et al., 1999). To investigate the effect of TNF on lymphoaccumulation, C57BL/6 *gld* TNF^{-/-} have been generated and TNF signaling was shown to amplify some aspects of the *gld* phenotype including lymphoaccumulation and premature death (Korner et al., 2000). In this scenario, TNF may induce the expression of chemokines that regulate the trafficking and accumulation of tumor cells into LNs.

To date there is little information to suggest that NK cells use TNF to directly exert anti-tumor activity, although TNF may contribute in effector cell migration to the tumor site. *In vivo* elimination of i.p. administered class I deficient RMA-S and RM-1 tumor is defective in TNF^{-/-} mice (Smyth et al., 1998). In this study, TNF was suggested to play a role in recruitment of NK cells into the peritoneum. TNF^{-/-} mice displayed reduced numbers of NK cells in the peritoneum that correlated with a defective NK cell anti-tumor response in these mice (Smyth et al., 1998). Similarly, TNF has also been shown to play a role in recruitment of NK cells to liver parenchyma (Pilaro et al., 1994).

Although TNF was originally discovered as a cytokine which had the ability to kill tumor cells, it is now evident that TNF can play a role in tumor promotion by regulating tumor cell proliferation, survival signals and stromal interactions which facilitate tumor invasion and metastasis (Frater-Schroder et al., 1987; Hideshima et al., 2001; Jourdan et al., 1999; Thomas et al., 1998). TNF is thought to exert this wide variety of functions by activating pathways involving NF- κ B, JNK and p38MAPK (Baud and Karin, 2001; Chen and Goeddel, 2002; Garg and Aggarwal, 2002; Migone et al., 2002; Pan et al., 1998a; Wajant et al., 2003).

1.3.4.3 FasL

FasL is a 40-kDa type II cell surface glycoprotein and member of the TNF-superfamily of ligands. FasL is expressed on activated T cells (Suda et al., 1993),

NK cells (Oshimi et al., 1996) and at sites of immune privilege (D'Alessio et al., 2001; French et al., 1996; Griffith et al., 1995; Hunt et al., 1997). FasL expression has been reported on a number of tumors (Hahne et al., 1996; O'Connell et al., 1996; Strand et al., 1996; Tanaka et al., 1996) and may play a role in 'tumor counterattack' (Maher et al., 2002) whereby FasL-expressing tumors eliminate Fas positive lymphocytes enabling the tumors to avoid rejection by the immune system. FasL has only one receptor, a 45-kDa type I transmembrane protein, Fas (CD95/Apo1) that is expressed on most normal and transformed cells.

Lpr (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mice, that have natural loss of function mutations in the *Fas* and *FasL* genes respectively (detailed in Chapter 1, Literature Review, Sections 1.7.2 and 1.7.3), exhibit an increased incidence of tumor development when backcrossed to E μ -myc or bcl-2 transgenic mice (Peng et al., 1996; Zornig et al., 1995), suggesting that a defective Fas/FasL pathway allows the accumulation of mutations that initiate tumor development, but alone are weak inducers of tumorigenesis.

Fas-sensitive melanomas have been demonstrated to metastasize to the lung in *gld* mice but not in wild-type (WT) mice (Owen-Schaub et al., 2000; Owen-Schaub et al., 1998) and IL-18-mediated suppression of tumor metastases in mice is predominantly mediated by FasL-expressing NK cells (Hashimoto et al., 1999). These data demonstrate the importance of the Fas/FasL pathway in the control of tumor spread.

Although high levels of Fas expression have been reported on some colon, ovary, breast, prostate and liver tumors (Nagata, 1996; Nagata and Golstein, 1995), many tumor cells do not constitutively express Fas. NK cells have been demonstrated to stimulate Fas expression on tumor cells *in vitro* and to subsequently induce Fas-dependent apoptosis in these targets (Screpanti et al., 2001). Tumor cells lacking Fas expression have also been shown to upregulate Fas when passaged *in vivo* (Screpanti et al., 2001). Expression of FLIP or CrmA in Fas-sensitive tumors has been shown to protect them from Fas-mediated

apoptosis, suggesting that FLIP and CrmA may function as tumor progression factors, protecting class-I deficient tumors from NK-cell mediated rejection *in vivo* (Screpanti et al., 2001; Seki et al., 2003). Collectively, these data indicate that FasL-mediated apoptosis might be an important means of NK cell-mediated tumor clearance.

Fas/FasL interactions are critical for homeostatic regulation of the immune system and play an important role in peripheral tolerance. *Lpr* and *gld* mice (detailed in Chapter 1, Literature Review, Section 1.7.2 and 1.7.3) develop lymphoaccumulation and have increased mortality due to an accumulation of B220⁺ DN T cells in LNs and spleen (Cohen and Eisenberg, 1991; Davidson et al., 1986) that are typically eliminated by Fas/FasL interactions. Fas/FasL interactions are also important in the activation induced cell death (AICD) of T cells (Ju et al., 1995; Kabelitz et al., 1993) that occurs after repeated CD3/TCR stimulation of the T cell and acts as a feedback mechanism to terminate ongoing immune responses. Similarly, activated T cells express both Fas and FasL, enabling them to kill each-other or commit suicide to dampen an immune response (Nagata and Golstein, 1995). Some reports suggest a role for Fas at high but not low intensity TCR stimulation in negative selection (Kishimoto et al., 1998). Together, these studies illustrate the significance of FasL-mediated apoptosis in T cell homeostasis.

The Fas/FasL pathway not only regulates T cell responses, but also plays a major role in elimination of activated B cells and may inhibit B cell tumor growth. Upon activation, B cells upregulate Fas and become sensitive to FasL-induced apoptosis (Daniel and Krammer, 1994; Onel et al., 1995; Stalder et al., 1994; Watanabe et al., 1995). FLIP-transduced A20 B cell tumor cells exhibit increased tumor growth *in vivo* compared with untransfected A20 cells, suggesting that Fas can control malignant B cell tumor growth *in vivo* (Djerbi et al., 1999). In addition, tumor cells isolated from a number of B cell malignancies are resistant to Fas-induced death even though they express adequate cell surface Fas receptor (Owen-Schaub et al., 1993; Plumas et al., 1998; Westendorf et al., 1995; Xerri et al., 1998). Moreover, FasL mutant *gld* mice develop B cell lymphomas with age

(Davidson et al., 1998) (detailed in Chapter 1, Literature Review, Section 1.7.2) and ALPS patients with Fas mutations are diagnosed with non-Hodgkin's and Hodgkin's B cell malignancies at 14 and 51 fold the normal incidences, respectively (Lin, 1995) (detailed in Chapter 1, Literature Review, Section 1.7.1). Together, these data suggest that Fas plays an important role in control of B cell tumor growth.

1.4 Recombinant (r)TRAIL/Apo2L

Recombinant soluble TRAIL is currently being developed as one of the most promising natural immune molecules proposed for trials in cancer patients. TRAIL shows particular promise given the unique ability of TRAIL to selectively induce death in cancer cells, but not in most normal cells. To date, several recombinant versions of human TRAIL have been generated and tested for their ability to induce tumor cell death *in vitro* and *in vivo* using murine cancer xenograft models. Initially, amino acids 114-281 of TRAIL were fused to an amino-terminal polyhistidine tag (Pitti et al., 1996). A second variant comprises amino acids 95-281 of TRAIL fused amino terminally to a modified yeast Gal-4 leucine zipper (LZ) that promotes trimerization of the ligand (Walczak et al., 1999). A third variant containing amino acids 95-281 fused to an amino-terminal 'Flag' epitope tag required crosslinking with an anti-Flag antibody for enhanced tumoricidal activity (Bodmer et al., 2000b; Chen et al., 2001a; Gong and Almasan, 2000). The most recent recombinant version of human TRAIL contains amino acids 114-281 without any added exogenous sequences (Ashkenazi and Dixit, 1999) decreasing the potential for immunogenicity in humans.

Both TNF and FasL have been shown to induce serious toxic effects after systemic administration (Galle et al., 1995; Hersh et al., 1991; Ogasawara et al., 1993; Timmer et al., 2002). There is also a concern that some rTRAIL variants may also induce systemic toxicity, highlighting the importance of preclinical assessment using this ligand. Polyhistidine tagged recombinant human TRAIL has been shown to induce apoptosis in normal human hepatocytes, but not in hepatocytes isolated from different species (Jo et al., 2000). Both recombinant

human LZ and polyhistidine tagged TRAIL have been shown to induce apoptosis in normal keratinocytes (Leverkus et al., 2000; Qin et al., 2001) and recombinant human LZ TRAIL is cytotoxic to human astrocytes *in vitro* (Walczak et al., 1999). In contrast, rTRAIL that lacks exogenous sequences does not induce apoptosis in normal human or cynomolgus monkey hepatocytes (Lawrence et al., 2001), human mammary, renal or prostatic epithelial cells, umbilical vein endothelial cells, lung fibroblasts, colon smooth muscle cells, astrocytes or keratinocytes and appears to be the most promising candidate for future clinical trials (Ashkenazi et al., 1999; Qin et al., 2001).

Production and purification of untagged rTRAIL has been optimized by the addition of zinc, the reducing agent dithiothreitol and a neutral pH, which ensures maximal stability of TRAIL homotrimers and activity (Kelley et al., 2001; Lawrence et al., 2001). Tagged versions of rTRAIL can aggregate and/or precipitate at high concentration and might therefore be able to induce apoptosis in normal cells by over-multimerizing DRs and surpassing the signal threshold required for activation of apoptosis in these cells (Ashkenazi, 2002; Lawrence et al., 2001). Untagged rTRAIL is largely homogeneous (99% trimer) and therefore cannot over-multimerize DRs in normal cells (Lawrence et al., 2001).

Preliminary studies using non-human primates (chimpanzees and cynomolgus monkeys) suggest that short-term (5-7 days) i.v. administration of untagged rTRAIL does not induce detectable toxicity (Ashkenazi et al., 1999; Lawrence et al., 2001). Although further animal safety studies need be undertaken to fully establish the effects of long term TRAIL administration, these preliminary toxicity studies provide real hope for the safe use of rTRAIL in clinical trials.

Like rTRAIL, a number of agonistic mAbs that functionally engage human and murine TRAIL receptors have been developed and investigated for anti-tumor potential. A number of agonistic mAbs to human DR4 [clones 4H6 (mouse IgG1) and 4G7 (mouse IgG2a)] (Chuntharapai et al., 2001) and DR4 and DR5 (Griffith et al., 1999a) have been developed, although these required cross-linking for activity. Agonistic mAbs that do not require cross-linking have also been

described for human DR5 [TRA-8 (mouse IgG1)] (Ichikawa et al., 2001) and DR4 and DR5 [anti-DR4 mAb (DR4-A) and anti-DR5 mAb (DR5-A) both mouse IgG1] (Wang et al., 2004). Only one agonistic mAb has been described for mouse DR5 (MD5-1) (Takeda et al., 2004). Unlike some forms of rTRAIL (Jo et al., 2000), the anti-human DR5 antibody (TRA-8) and anti-mouse DR5 mAb have been reported to lack hepatotoxicity (Ichikawa et al., 2001; Takeda et al., 2004).

1.4.1 Anti-tumor activity

rTRAIL can induce apoptosis in a wide variety of human cancer cell lines, including; breast, bladder, ovary, lung, prostate, colon, kidney, CNS, pancreatic and hematopoietic cells (Ashkenazi et al., 1999; Chen et al., 2001a; Gazitt, 1999; Keane et al., 1999; Lincz et al., 2001; Liu et al., 2001; Mitsiades et al., 2001a; Mitsiades et al., 2001b; Mitsiades et al., 2002; Mitsiades et al., 2001c; Mizutani et al., 1999; Rieger et al., 1998; Snell et al., 1997; Thomas and Hersey, 1998; Walczak et al., 1999; Yu et al., 2000). *In vitro*, Ashkenazi and colleagues demonstrated that rTRAIL had a cytostatic effect on 16 of 39 tumor cell lines tested, inhibiting cell growth by 50-100% (Ashkenazi et al., 1999). The same researchers observed that rTRAIL exerted a cytotoxic effect on 16 additional tumor cell lines, reducing cell numbers by 50-100% (Ashkenazi et al., 1999).

Both i.p. and i.v. modes of rTRAIL administration have been shown to inhibit tumor growth with the anti-tumor effect of TRAIL appearing dose-dependent (Walczak et al., 1999). Recombinant human and murine LZ TRAIL lyse TRAIL-sensitive murine LB27.4 tumor cells equivalently, although the TRAIL-sensitive human Jurkat cell line is slightly more sensitive to lysis by human LZ TRAIL compared with murine LZ TRAIL (Walczak et al., 1999). These studies suggest a significant cross-species activity for rTRAIL, permitting extensive preclinical assessment of this protein in animal models.

In vivo, rTRAIL has demonstrated remarkable anti-tumor activity against colon carcinoma (Ashkenazi et al., 1999; LeBlanc et al., 2002), glioma (Fulda et al., 2002; Pollack et al., 2001), multiple myeloma (Mitsiades et al., 2001a) and breast

carcinoma xenografts (Walczak et al., 1999), in established models of cancer. Furthermore, rTRAIL induced apoptosis in established xenografts (Ashkenazi et al., 1999; Walczak et al., 1999). Moreover, cells isolated from tumors that re-emerge after an initial regression following TRAIL administration remain TRAIL-sensitive *in vitro*, suggesting that further treatment with rTRAIL might eliminate these tumors (Walczak et al., 1999). Not only can rTRAIL suppress the growth of cell-line based cancer xenografts in mice, but also xenografts of human tumor cells isolated from patients and grown only briefly in culture before transplantation into mice (Ashkenazi, 2002).

Several *in vitro* studies have demonstrated the cytotoxic activity of agonistic DR4 and DR5 mAbs against tumor targets *in vitro*, although receptor cross-linking has been required in a number of these studies (Chuntharapai et al., 2001; Griffith et al., 1999a; Ichikawa et al., 2001; Takeda et al., 2004). A number of tumor xenograft studies have also demonstrated the capability of agonistic mAbs that engage human DR4 or DR5 to exert anti-tumor activity *in vivo* (Chuntharapai et al., 2001; Ichikawa et al., 2001; Wang et al., 2004). A mAb to murine DR5 (MD5-1) has recently been reported to inhibit spontaneous 4T1.2 and R331 lung and liver metastasis *in vivo* by recruiting Fc receptor-expressing innate immune cells (Takeda et al., 2004). The anti-metastatic effect of MD5-1 was inhibited for FLIP-transfected R331 tumors, suggesting that the anti-metastatic effects of MD5-1 are primarily mediated by caspase-dependent signaling via DR5 rather than complement-mediated lysis or ADCC (Takeda et al., 2004). Together, these studies illustrate that the TRAIL pathway can be manipulated not only by administration of rTRAIL, but also agonistic TRAIL receptor mAbs to inhibit tumorigenesis.

A recent study by Wang and colleagues demonstrated that overexpression of the *MYC* oncogene in a number of human cell types of epithelial and mesenchymal origin sensitized them to apoptosis induced by rTRAIL and TRAIL death receptor (DR5) agonists (Wang et al., 2004). Myc sensitized cells to TRAIL/DR5 agonist mediated apoptosis by upregulating cell surface expression of DR5 and stimulating autocatalytic processing of pro-caspase 8. DR5 agonists were also

demonstrated to inhibit the growth of Myc-expressing tumor xenografts *in vivo* (Wang et al., 2004).

1.4.2 Synergy with chemotherapy and radiotherapy

p53 is commonly inactivated in progressing human cancers and in patients receiving conventional treatments such as chemotherapy and radiotherapy. Radiation and most DNA-damaging chemotherapeutic drugs require p53 function for activity (Levine, 1997) and are ineffective in these patients. TRAIL can induce apoptosis in transformed cells regardless of their p53 status (Ashkenazi et al., 1999), providing a unique therapeutic strategy to circumvent resistance to therapy in patients lacking p53 function (El-Deiry, 2001; Sheikh et al., 1998).

p53 is known to regulate *DR5* gene transcription (Wu et al., 1997), raising the possibility that p53-activating agents may act in synergy with rTRAIL by upregulating DR5 expression and sensitizing cells to TRAIL-mediated apoptosis. This knowledge has stimulated research focused on the investigation of combined rTRAIL and chemotherapeutic drug administration. TRAIL has been shown to act in synergy with radiation (Chinnaiyan et al., 2000; Gong and Almasan, 2000) and chemotherapeutic drugs such as adriamycin, cisplatin, cis-diaminedichloroplatinum (II) (CDDP), actinomycin-D, doxorubicin, etoposide, camptothecin, fluorouracil and lomustine (Ashkenazi et al., 1999; Bonavida et al., 1999; Gliniak and Le, 1999; Keane et al., 1999; Mizutani et al., 1999; Morrison et al., 2002; Nagane et al., 2001; Nagane et al., 2000; Olsson et al., 2001; Wen et al., 2000) in a variety of cancers including bladder, glioma, colon and breast. Upregulation of DR5 expression or downregulation of cFLIP expression by these drugs may be responsible for enhanced TRAIL activity in these *in vitro* and *in vivo* studies.

Xenogeneic mouse tumor models have also been used to demonstrate the synergy between TRAIL and chemotherapeutic drugs. TRAIL combined with 5-fluorouracil or camptosar acts synergistically to cause regression and even remission of established human colon carcinomas in xenografted nude mice

(Ashkenazi et al., 1999) and CDDP co-administered with TRAIL acts synergistically to suppress formation and growth of established human glioblastoma xenografts in nude mice (Nagane et al., 2000). Some mismatch-repair (MMR)-deficient tumors evade TRAIL-mediated apoptosis by inactivating Bax, and thus the 'cell-intrinsic' TRAIL apoptotic pathway. Recent studies have demonstrated that a combination of TRAIL and chemotherapeutic agents can restore TRAIL sensitivity in Bax^{-/-} human colon cancer cells (LeBlanc et al., 2002; Wang and El-Deiry, 2003). This restoration of TRAIL sensitivity was demonstrated to be p53-dependent, with p53-stimulation of DR5 expression playing an important role in the process (Wang and El-Deiry, 2003).

1.5 The thymus

The thymus is a bi-lobed organ found in the thorax in the anterior mediastinum and is the site of mainstream T-cell development, where BM-derived stem cells undergo a series of maturation events and exit as self-MHC restricted, self-tolerant, naïve T cells. Each thymic lobe comprises numerous lobules separated by fibroblast-rich trabeculae that extend inwards from the subcapsule. These lobules can be further divided into two major regions, an outer cortex that hosts early thymocyte development and an inner medulla that is important for late T cell development (Boyd et al., 1993). For the purposes of this literature review, an overview of T cell development is presented, with a focus on negative selection, as this area is most relevant to this thesis.

In addition to T, B and NKT lymphocytes, the thymus is also comprised of a non-lymphocytic component broadly termed the thymic stroma. The thymic stroma is made up of cells of endodermal, ectodermal and mesodermal origin (endothelial, epithelial and mesenchymal cells and fibroblasts) as well as macrophages and DCs (Boyd et al., 1993). Together, these cells provide a variety of known and as yet undefined soluble factors, extracellular matrix elements and cell surface molecules that are essential for various stages of T-cell development (Anderson et al., 1996; Shortman and Wu, 1996).

Hematopoietic stem cells from the BM enter the thymus in a narrow region of the perimedullary cortex and migrate outwards across the cortex to accumulate in the subcapsular zone (SCZ) (Lind et al., 2001). Migration of the blood-derived precursors between different regions of the cortex correlates with defined stages of early lymphoid development (Lind et al., 2001). Four phenotypically distinct stages, based on expression of CD25 (IL-2-R α chain) and the adhesion molecule CD44 (Godfrey and Zlotnik, 1993) are used to describe the maturation state of cells in this region. In order of increasing maturity, these are CD25⁻CD44⁺ [double negative (DN)1], CD25⁺CD44⁺ (DN2), CD25⁺CD44⁻ (DN3) and CD25⁻CD44⁻ (DN4) (Godfrey et al., 1993). Broadly speaking, these cells are CD3^{-/lo}, CD4^{-/lo} and CD8⁻.

The DN1 population contains cells with multi-lineage potential and requires IL-7 and stem cell factor for continued development (Di Santo and Rodewald, 1998; Rodewald et al., 1997). The DN1 population differentiates into DN2 cells that are thought to commence TCR- γ and TCR- β gene rearrangement as they transit from the DN2 into the DN3 compartment (Dudley et al., 1994; Godfrey et al., 1994). Following recombination of the TCR- β locus, the TCR- β chain assembles with the pre-TCR- α (pT α) protein and the CD3 complex (Fehling and von Boehmer, 1997). In a process termed β -selection, signaling through the TCR- β -pT α -CD3 complex via CD3 and lck (Borowski et al., 2004; Saint-Ruf et al., 2000) then stimulates the cells to proceed through the DN4 to the CD4⁺CD8⁺ double positive (DP) stage of thymocyte maturation, to undergo proliferation and to commence TCR- α chain gene rearrangement (Dudley et al., 1994). Prior to export from the thymus, DP thymocytes undergo thymic selection to ensure that the majority of thymocytes entering the periphery are capable of interacting with self-MHC with low affinity for self-antigens (Figure 1.9).

1.5.1 Mechanisms of thymic selection

DP thymocytes that express TCRs with a low affinity for peptide-MHC undergo positive selection (Anderson et al., 1993) and can exit to the periphery where they

circulate throughout the secondary lymphoid organs awaiting encounter with antigen (Goldrath and Bevan, 1999). DP thymocytes that express TCRs with a high affinity for self peptide-MHC undergo negative selection, leading to apoptosis (Palmer, 2003). Approximately 90% of all DP thymocytes fail positive selection and undergo apoptosis due to 'death by neglect' (Huesmann et al., 1991) (Figure 1.9). This form of death occurs when thymocyte $\alpha\beta$ TCR fails to engage a peptide-MHC ligand with sufficient avidity to provide appropriate survival signaling. Collectively, the positive and negative selection processes ensure the generation of a largely self-MHC restricted, self-tolerant peripheral T cell repertoire.

Approximately 5% of total DP thymocytes (pre-selection) are thought to recognize peptide-MHC ligands with a low affinity and undergo positive selection (van Meerwijk et al., 1997). It is now clear that a low affinity MHC interaction alone is not sufficient for positive selection to occur, as while cortical epithelial cells can mediate positive selection, MHC expressing stromal cells from other tissues cannot (Anderson et al., 1994). This suggests that an as yet undiscovered co-receptor present only on cortical stroma may be required to help stimulate positive selection *in vivo* (Chidgey and Boyd, 2001; Hare et al., 2002). DP thymocytes that receive a sustained positive selection signal (Wilkinson et al., 1995) upregulate CD69 and TCR, downregulate RAG expression and migrate from the thymic cortex to the medulla. In the medulla, SP thymocytes continue to differentiate into a population with a phenotype that closely resembles peripheral T cells (downregulated CD69 and HSA and upregulated CD62L and Qa2) (Gabor et al., 1997; Lucas et al., 1994; Wallace et al., 1992).

Two distinct models have been proposed for the commitment of DP thymocytes to the CD4 or CD8 lineage (Germain, 2002). The instructive model proposes that interaction between CD4 or CD8 co-receptors, with peptide-MHC class I or II respectively, induces commitment of the DP thymocyte to the CD4 or CD8 lineage and inhibits transcription of the 'inappropriate' co-receptor. The stochastic model suggests that DP thymocytes are either pre-committed to the CD4 or CD8 lineage or make a lineage choice at the start of positive selection

independently of the specificity of their TCR. In this model, after one co-receptor is lost, further T cell maturation is dependent on signals that require a match between the MHC-class specificity of the remaining co-receptor and the cell's TCR selection.

Negative selection is essential for deletion of potentially self-reactive thymocytes and the generation of a largely self-tolerant peripheral T cell repertoire. Approximately 5% of total DP thymocytes (pre-selection) are believed to express a TCR potentially capable of recognizing self-peptide-MHC ligands with sufficiently high affinity that they would be a potentially autoreactive T cell clone. These cells are instead targeted for negative selection (van Meerwijk et al., 1997). Depending on the model system used, negative selection appears to occur at the immature DP stage of thymocyte differentiation and/or later at the single positive (SP) HSA^{hi} semi-mature stage when thymocytes transit through the CMJ (Stockinger, 1999) (Figure 1.9). Mature SP HSA^{lo} thymocytes within the medulla appear to be functionally similar to naïve T cells in the periphery and are generally resistant to negative selection (Kishimoto and Sprent, 1997).

In addition to a high affinity MHC interaction, negative selection is thought to require an additional co-stimulation signal(s), although the identity of such a signal remains elusive (Page et al., 1993; Punt et al., 1994). A number of groups have used activating antibodies to co-stimulatory molecules to investigate the role of these molecules in negative selection. Activating antibodies to CD28 can co-stimulate apoptosis induction in DP thymocytes (Kishimoto and Sprent, 1999; Punt et al., 1994) suggesting a potential role for this molecule in negative selection. Similarly, activating antibodies to CD28, CD5, and CD43 have been reported to enhance apoptosis of semi-mature (CD4⁺ SP HSA^{hi}) thymocytes (Kishimoto and Sprent, 1999). Another report used blocking antibodies to illustrate a role for CD5, CD80, CD86 and TNF in co-stimulation of negative selection (Page, 1999). In that study, addition of blocking antibodies to FTOC rescued the CD4⁺ SP thymocytes from negative selection (Page, 1999).

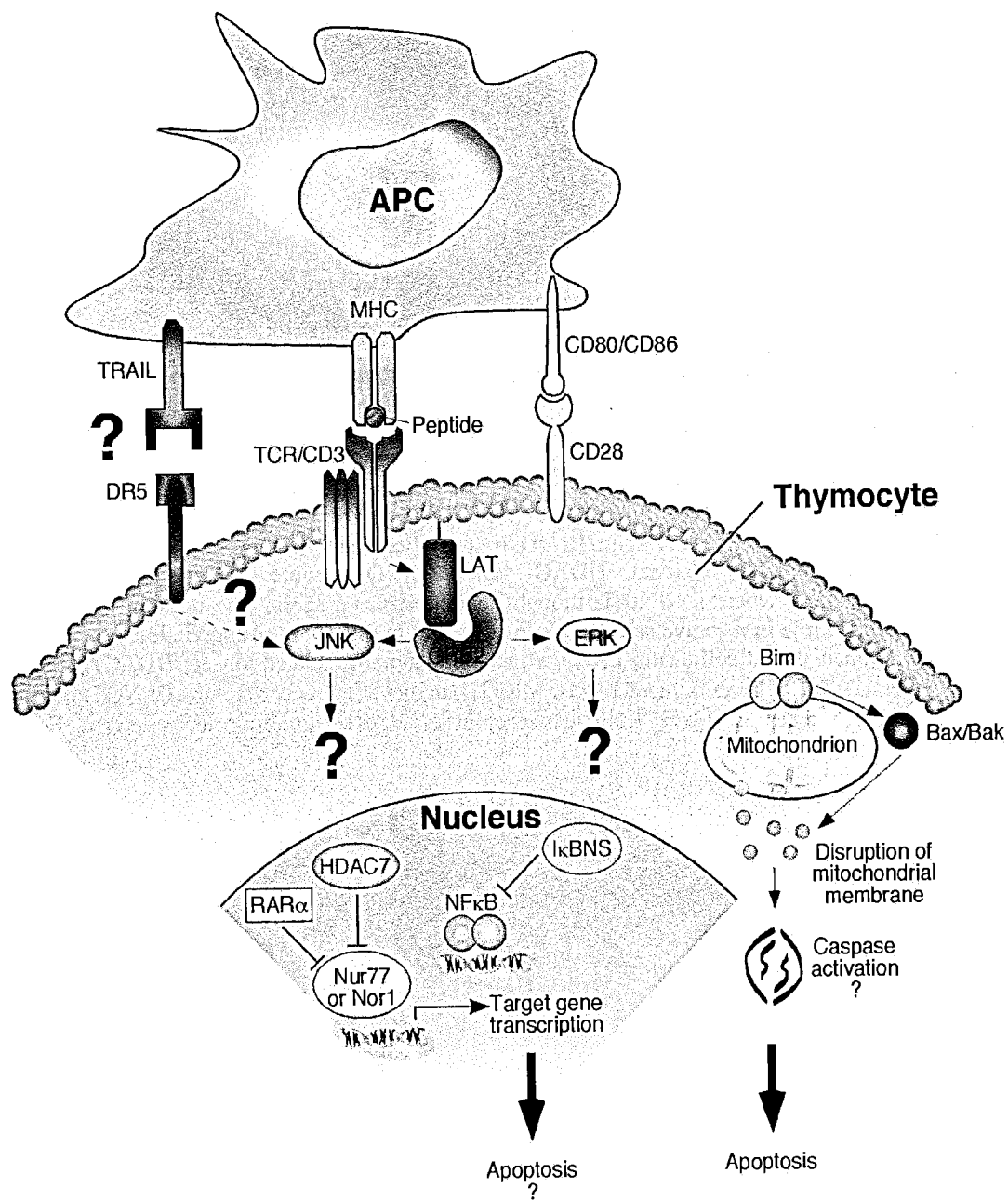
In contrast to the *in vitro* antibody studies that suggested that CD28, CD5, CD86 and CD80 can co-stimulate negative selection, CD28^{-/-}, CD5^{-/-}, CD5^{-/-}CD28^{-/-} and CD80^{-/-}CD86^{-/-} mice exhibited normal negative selection (Li and Page, 2001; Walunas et al., 1996; Williams et al., 2002). These discordant results suggest a redundant role for these molecules *in vivo*, or alternatively the requirement for co-stimulation might be dependent on the affinity of the TCR signal received.

1.5.2 Signaling factors critical for negative selection

The search for the apoptosis signaling pathway(s) in thymocyte negative selection has been a long-term quest for T cell immunologists. Both the DR pathway (Shi, 2002) (detailed in Chapter 1, Literature Review, Section 1.5.3) and the mitochondrial/bcl-2 regulated pathway triggered by various stress stimuli, such as developmental signals and growth factor deprivation (Strasser et al., 2000; Wang, 2001), have been implicated in thymocyte negative selection. Although the signaling pathway(s) that mediate negative selection are not fully understood, a number of factors have been identified (Figure 1.10).

Various studies support a role for the MAPK pathway in negative selection. High affinity engagement of the TCR with peptide-MHC ligand leads to the phosphorylation of linker for activation of T cells (LAT). Phosphorylation of the tyrosine at position 136 has been hypothesized to play a role in negative selection, as mutation of this tyrosine to a phenylalanine results in autoimmune disease development in mice (Aguado et al., 2002; Sommers et al., 2002). Phosphorylated LAT recruits growth-factor receptor-bound protein 2 (GRB2) that is thought to be important for negative but not positive selection (Gong et al., 2001) resulting in activation of MAPK signaling pathways such as JNK and p38. CD3 mAb-induced apoptosis of JNK2^{-/-} thymocytes is partially defective (Sabapathy et al., 2001), JNK1 dominant-negative mice have defects in apoptosis following administration of negative-selecting peptide (Rincon et al., 1998) and a p38 inhibitor interferes with negative selection in FTOC experiments (Sugawara et al., 1998). Although these experiments implicate the MAPK signaling pathway in negative selection, further experiments are required to determine the interplay

Figure 1.10. Negative selection signaling pathways. A number of signaling pathways have been implicated in thymocyte negative selection. High affinity engagement of the TCR with peptide-MHC ligand, coupled with co-stimulation begins the process. This initiates the activation of downstream MAPK signaling pathways that are known to play a role in negative selection. The pro-apoptotic Bcl-2 family proteins Bim, Bax and Bak are also key mediators of negative selection and may lead to death of autoreactive thymocytes by activating caspases. Within the nucleus, members of the Nur77/Nur1/Nor1 transcription family, that are under the control of the upstream regulatory elements HDAC-7 and MEF2D (Dequiedt et al., 2003), promote negative selection, whereas NF κ B is thought to inhibit the process. The question of whether TRAIL plays a role in negative selection is investigated in this study. Abbreviations: Bim, Bcl-2 interacting mediator of cell death; ERK, extracellular-signal-regulated kinase; HDAC7, Histone deacetylase 7; I κ BNS, inhibitor of NF κ B; MEF2D, myocyte enhancer factor 2D; Nor1, neuron-derived orphan receptor 1; Nur77, nuclear receptor 77; RAR α , retinoic-acid receptor α . Figure courtesy of Adam Uldrich, adapted from (Palmer, 2003).



between MAPK signaling pathways and other signaling cascades that ultimately lead to induction of negative selection.

Both RNA and protein synthesis are important for clonal deletion (Osborne et al., 1994). Members of the Nur77/Nurr1/Nor1 transcription family promote negative selection, whereas NF κ B is thought to inhibit the process. Interestingly, Nur77 does not appear to be involved in positive selection, highlighting that positive and negative selection are distinct processes and not outcomes of the one signaling pathway (Amsen et al., 1999). Stimulation of the TCR in thymocytes activates Nur77 transcription and negative selection is inhibited in thymocytes isolated from Nur77 dominant-negative mice (Calnan et al., 1995) suggesting an important role for this transcription factor in clonal deletion. Nur77 and family member Nor-1 have been shown to be structurally and functionally redundant (Cheng et al., 1997), which might explain the lack of a negative selection defect in Nur77^{-/-} mice (Lee et al., 1995). Within the nucleus, I κ BNS inhibits NF κ B-mediated transcription promoting negative selection (Fiorini et al., 2002). Furthermore, transcription of I κ BNS is stimulated by peptides that induce negative selection and DP thymocytes that are sensitive to negative selection lack NF κ B transcriptional activity (Simon et al., 2000).

Death induced by the mitochondrial cell death pathway involves members of the Bcl-2 family of proteins. In resting cells, anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-x_L, Bcl-w, Boo, A1, Bcl-B and Mcl-1) sequester pro-apoptotic 'BH3-only' Bcl-2 family members such as Bim, Bad, Bid, Bik and Bmf (Puthalakath and Strasser, 2002) preventing them from inducing apoptosis. Upon receipt of an apoptotic signal, the 'BH3-only' proteins are released, allowing them to activate a third group of Bcl-2 family members, the pro-apoptotic 'multidomain' or 'BH123' proteins (Bax, Bak and others). Bax and Bak can then permeabilize the mitochondrial outer membrane, stimulating the release of cytochrome c. Cytochrome c liberated from the mitochondria then binds to the adaptor protein APAF-1, forming the apoptosome and activating caspase-9 which in turn then activates executioner caspases leading to target cell death (Green, 2003).

The pro-apoptotic Bcl-2 family member Bim is critical for thymic negative selection. Bim-deficient thymocytes are protected from negative selection induced by TCR-CD3 stimulation *in vitro* and *in vivo*, stimulation with the superantigen SEB *in vitro* or *in vivo* and injection with antigenic peptide *in vivo* (Bouillet et al., 2002; Villunger et al., 2004). Moreover, Bim is required for the deletion of autoreactive HY male antigen-specific thymocytes in HY-TCR transgenic mice (Bouillet et al., 2002). The pro-apoptotic molecules Bax and Bak, required by Bim to elicit apoptosis (Zong et al., 2001), also play a role in clonal deletion. Thymocytes deficient in both Bax plus Bak are resistant to negative selection induced by TCR-CD3 stimulation *in vitro* and endogenous retroviral superantigens *in vivo* (Rathmell et al., 2002). Together, these studies illustrate the importance of the mitochondrial/bcl-2 regulated pathway in negative selection.

1.5.3 Role of the TNF superfamily in negative selection

Members of the TNF superfamily play a well established role in T cell apoptosis within peripheral tissues, however, their role in the thymus is less clear. A multitude of these receptors and ligands (including: Fas, TNF, LT, LIGHT, DR3, CD30) have been investigated for their role in negative selection (DeYoung et al., 2000; Page et al., 1998; Sprent and Kishimoto, 2002; Wang et al., 2001a; Wang et al., 2001c). Blockade of the co-stimulatory molecule LIGHT using LT β R-Ig inhibited negative selection of thymocytes, and apoptosis was increased in the thymus of LIGHT transgenic mice suggesting that LIGHT plays a role in thymic negative selection (Wang et al., 2001c). The role of CD30 in negative selection has been controversial, with one report suggesting that CD30^{-/-} mice have impaired negative selection of thymocytes (Amakawa et al., 1996) and another suggesting that negative selection is normal in these mice (DeYoung et al., 2000). Moreover, one study suggested that CD30 acts as a co-stimulatory molecule for negative selection (Chiarle et al., 1999), whilst another, using an activating anti-CD30 antibody suggested that CD30 does not provide co-stimulatory activity for apoptosis of DP thymocytes *in vitro* (Kishimoto and Sprent, 1999). Further detailed studies of CD30^{-/-} mice are needed to fully determine the role of CD30 in negative selection.

The TNF superfamily DRs have also been investigated. TNF blockade prevented the negative selection of CD4⁺ SP thymocytes in FTOC (Page, 1999), although normal negative selection has been reported in TNF^{-/-} mice (Grech et al., 2000). Curiously, TNFR^{-/-} mice displayed impaired negative selection in some models and normal negative selection in others (Page et al., 1998). Conflicting results are also evident for DR3, the receptor for TL1A. DR3-deficient mice exhibit normal negative selection in some models, but impaired negative selection in others (Wang et al., 2001a). A role for Fas has been reported at high, but not low, intensity TCR stimulation (Kishimoto et al., 1998). LT- α has also been investigated, but does not appear to play a role in clonal deletion (Grech et al., 2000). Furthermore, studies by Newton and colleagues and Smith and colleagues suggest that negative selection occurs normally in FADD dominant-negative mice (Newton et al., 1998) and in transgenic mice lacking caspase-8 function (Smith et al., 1996). Given that the DRs utilize these pathways to induce apoptosis, one might expect that these receptors do not play a role in negative selection. These discrepancies are difficult to explain and further studies are needed to explain the role of the DRs in negative selection.

1.5.4 Link between negative selection defects and autoimmune disease

A breakdown of immunological tolerance to self-tissue may lead to the development of autoimmune disease. As negative selection is responsible for the removal of potentially autoreactive thymocytes during development, defects in negative selection might allow the release of self-reactive T cells into the peripheral pool, predisposing an individual to autoimmune disease.

A direct relationship between failures of negative selection and the release of autoreactive T cells from the thymus has been demonstrated in a number of different models. For example, failure to delete autoreactive thymocytes, due to mutation of the pro-apoptotic molecule Bim, generally leads to the development of spontaneous autoimmune glomerulonephritis (Bouillet et al., 1999). Autoimmune regulator (AIRE) is a transcription factor expressed in rare thymic

stromal cells that is thought to promote the expression of self-antigens whose expression is otherwise limited to the periphery (Heino et al., 1999). AIRE-dependent expression of peripheral antigens in the thymus is presumed to lead to the deletion of self-reactive T cells, presumably through negative selection. Thus, defects in AIRE function may disturb this process, allowing the persistence of autoreactive T cells that would typically be negatively selected. Consistent with this, AIRE-deficient mice exhibit reduced ectopic expression of peripheral antigens by thymic epithelial cells in the medulla and develop a spectrum of organ-specific autoimmunity (Anderson et al., 2002). Similarly, patients expressing a defective form of AIRE often develop a multi-organ autoimmune disease called autoimmune polyendocrine syndrome type 1, consistent with a loss of tolerance to a variety of peripheral self-antigens (Nagamine et al., 1997). Moreover, autoreactive CD4⁺ T cells with high affinity for a transgenically-expressed pancreatic antigen were normally deleted in the thymus, but when mice were backcrossed to an AIRE-deficient background, negative selection of the developing cells was impaired (Liston et al., 2003). These studies are the first direct evidence that autoimmunity, as opposed to the simple survival of self-reactive T cells, can be induced by a defect in negative selection.

1.6 Autoimmune disease

Autoimmune disease results from a breakdown of immunological tolerance to self-antigens and includes diseases of the nervous, gastrointestinal, and endocrine systems, as well as skin and other connective tissues, eyes, blood, and blood vessels. Approximately 75 percent of autoimmune diseases occur in women, most frequently during the childbearing years (American Autoimmune Related Diseases Association). Maintenance of self-tolerance is achieved by a number of mechanisms including; negative selection (detailed in Chapter 1, section 1.5.2 and 1.5.3), suppression of autoreactive lymphocytes and functional inactivation (anergy). Members of the TNF superfamily are potent inducers of apoptosis and have been implicated in the regulation of autoimmune disease. Much of what is understood of the pathophysiology of autoimmune disease can be attributed to the development of experimental models of autoimmune disease in animals.

1.6.1 Role of TNF superfamily molecules in autoimmune disease

The TNF superfamily has been described as representing a double-edged sword (Aggarwal, 2003): whilst they are essential for normal physiological responses, inappropriate expression and function can be harmful as illustrated by their involvement in autoimmune disease. Over the last decade, much research has focused on understanding the role of the TNF superfamily cytokines and receptors in experimental models of autoimmune disease. Most of these studies have focused on dissecting the roles that TNF, Fas, TRAIL and LT- α play in EAE, autoimmune arthritis and autoimmune diabetes. Whilst TRAIL has been shown by a number of research groups to inhibit autoimmune disease in a variety of animal models, the function of other TNF superfamily members and the roles they might play in the control of autoimmune disease has been more controversial. These conflicting results may indicate the complexity of involvement of the TNF superfamily receptors and cytokines in autoimmune disease.

Drawing a consensus from the literature regarding the roles for various TNF superfamily members in autoimmune disease is difficult. TNF exacerbates a number of autoimmune diseases by contributing to important inflammatory processes. TNF inhibitors ameliorate arthritis in mice by inhibiting joint inflammation and damage (Joosten et al., 1996; Maini et al., 1995; Williams et al., 1992) and are successfully being used to treat rheumatoid arthritis in humans (Maini et al., 1995). TNF-blockade in patients with Crohn's disease significantly decreases inflammation of the mucosa that is associated with disease (D'Haens, 2003). TNF also plays a critical role in the CNS inflammatory process of mice immunized to develop EAE (detailed in Chapter 1, Literature Review, Section 1.6.3.1.1) (Selmaj et al., 1995; Selmaj et al., 1991).

The role that FasL plays in autoimmune disease is complex and might be explained if FasL plays a dual role in the disease process. For example, FasL appears to play a role in CNS destruction during the initiation stage of EAE but during the recovery stage regulates autoreactive T cell activity (detailed in

Chapter 1, Literature Review, Section 1.6.3.1.3) (Dittel et al., 1999; Sabelko-Downes et al., 1999). FasL-mediated apoptosis of T and B cells appears to commonly play a role in autoimmune disease. For example, mice and humans with mutations in *Fas/FasL* genes develop systemic autoimmune diseases and produce high levels of autoantibodies (Bettinardi et al., 1997; Cohen and Eisenberg, 1991; Sneller et al., 1997) that may typically be inhibited by FasL-mediated apoptosis of autoreactive T cells and antibody producing autoimmune B cell clones respectively (Rieux-Laucat et al., 2003).

TRAIL has consistently been shown to inhibit autoimmune disease in a number of animal models, although the role it plays in each disease appears to vary. Studies using TRAIL^{-/-} mice, soluble TRAIL receptor (sDR5) to block TRAIL function or TRAIL adenovirus have demonstrated that TRAIL inhibits autoimmune arthritis (Lamhamedi-Cherradi et al., 2003b; Song et al., 2000) and cyclophosphamide and streptozotocin-induced diabetes in mice (Lamhamedi-Cherradi et al., 2003a; Lamhamedi-Cherradi et al., 2003b). In these studies, TRAIL was suggested to play a variety of roles ranging from inhibiting cytokine and antibody production to inhibiting inflammation and cell cycle progression. More recent studies have demonstrated that induction of TRAIL expression on collagen-pulsed DCs can suppress autoimmune arthritis in mice, offering new and exciting possibilities for cell-based treatment of patients with autoimmune disease (Liu et al., 2003).

1.6.2 Multiple Sclerosis (MS)

MS is the most common neurological disease affecting young adults today. The average age of onset for MS is between 20-40 years of age and women are more commonly affected than men (Duquette et al., 1992; Lowis, 1990; Sadovnick and Ebers, 1993). MS is an autoimmune disease that involves both cellular and humoral responses directed against proteins of the myelin sheath, such as MOG, myelin basic protein (MBP) and proteolipid protein (PLP) (Abo et al., 1993; Mokhtarian et al., 1984; Schluesener et al., 1987; Steinman, 1996). The disease is characterized by an infiltration of inflammatory cells into the CNS, accompanied

by localized destruction of the myelin sheath and loss of oligodendrocytes that form the myelin sheath (Ewing and Bernard, 1998). MS lesions contain activated CD4⁺ T cells that secrete a variety of cytokines such as IFN- γ and TNF- α , B cells and macrophages and most patients have oligoclonal IgG bands with a specificity for myelin proteins in their cerebrospinal fluid (Ewing and Bernard, 1998; Raine, 1991). Myelin-specific T cells are present in healthy individuals as well as MS patients, suggesting that T cell regulatory mechanisms may be defective in affected individuals (Markovic-Plese et al., 2001; Pette et al., 1990).

MS symptoms include, paresis and paralysis, ataxia, fatigue, impaired vision, sensations of burning or prickling, cognitive impairment and incontinence (Camp et al., 1999; Tienari, 1994). Although the disease course is highly variable, most MS patients can be classed as developing either chronic progressive MS (CP-MS) or relapsing-remitting MS (RR-MS) (Tienari, 1994). The cause of MS is not known, although a genetic component exists (family members of MS patients have a higher probability of developing the disease) (Doolittle et al., 1990), environmental factors play a role (there is an increased incidence of MS with latitude away from the equator) (McLeod et al., 1994) and significant variations exist in some ethnic groups (Kurtzke, 1985). It is generally believed that the autoreactivity associated with MS is caused by either a failure of self-tolerance, or molecular mimicry, where microbe-specific T cells cross the blood-brain barrier and cross-react with myelin proteins (Ewing and Bernard, 1998).

1.6.2.1 TRAIL and MS

Most therapies for MS have focused on the use of steroids to suppress the inflammatory response associated with the disease (Griffiths and Newman, 1994). Perhaps the most promising therapy currently available for MS patients is IFN- β . IFN- β was the first drug approved by the US-FDA for the treatment of MS and has been shown to decrease the rate of clinical relapse in patients and delay disability (Ruuls and Sedgwick, 1998). The mechanism by which IFN- β inhibits disease is currently being investigated, but recently TRAIL has been identified as a potential response marker for IFN- β therapy (Wandinger et al.,

2003). Patients with raised levels of soluble TRAIL in their sera prior to IFN- β therapy and those who showed early and sustained TRAIL induction after therapy were most likely to respond to treatment (Wandinger et al., 2003). PBLs isolated from MS patients have also been reported to express higher levels of TRAIL than healthy control subjects (Huang et al., 2000), but it has yet to be investigated whether levels of PBL TRAIL expression correlate with response to IFN- β therapy.

Researchers have shown that although the death-inducing TRAIL receptors TRAIL-R1 and TRAIL-R2 are expressed on human MBP-specific T cells lines derived from MS patients and healthy donors, these cells were not susceptible to TRAIL-mediated apoptosis induced by rTRAIL (Wendling et al., 2000). In fact, upon activation of these T cells with anti-CD3 and anti-CD28, expression of TRAIL-R1 and TRAIL-R2 actually decreased whereas TRAIL expression was increased on these cells (Wendling et al., 2000). These data suggest that the role of the TRAIL receptors expressed on autoreactive T cells in MS patients might not involve regulation of T cell sensitivity to death.

1.6.3 Experimental autoimmune encephalomyelitis (EAE)

EAE is an animal model with many clinical and histopathological similarities to MS. EAE can be induced in a variety of animal species including; mouse, rat, guinea-pig and monkey. Like MS, EAE is characterized by an early breach of the blood-brain barrier, infiltration of inflammatory cells into the CNS and demyelination that ultimately leads to paralysis of the animal (Bernard et al., 1992; Raine, 1990).

In mice, EAE can be induced by immunization with myelin or myelin components, including MBP, PLP or myelin peptides such as MOG. EAE is also induced by adoptive transfer of MBP, PLP, or MOG-specific CD4⁺ Th1 T cells into naïve recipients. TCR transgenic mice that are specific for MBP, PLP and MOG have also been generated (Lafaille et al., 1994; Mendel et al., 2004; Waldner et al., 2000). PLP transgenic mice developed spontaneous EAE

compared with only a proportion of the MBP transgenic mice, although 100% of the MBP transgenic mice developed spontaneous disease when crossed to RAG-1-deficient mice. MOG transgenic mice require peptide immunization in order to develop the disease.

Immune responses to MOG have been identified as critical for the development of demyelinating forms of EAE (Lebar et al., 1986), which might not be surprising, given that MOG is an oligodendrocyte transmembrane protein present on the outermost lamellae of the myelin sheath (Brunner et al., 1989; Lebar et al., 1986; Linnington et al., 1984). MOG-specific antibodies, but not PLP or MBP-specific antibodies can mediate demyelination *in vitro* and *in vivo* (Kerlero de Rosbo et al., 1990; Linnington et al., 1988; Linnington et al., 1992; Piddlesden et al., 1993; Schluesener et al., 1987). The immunodominant T cell responses to MOG in rodents are to sequences 1-20 and 35-55 (Bernard et al., 1997; Linnington et al., 1993; Slavin et al., 1998). MOG₃₅₋₅₅ peptide induces a relapsing-remitting form of EAE in NOD/Lt mice and a chronic non-remitting paralytic disease in C57BL/6 mice, which is characterized by MNC infiltration into the CNS and multifocal demyelination in the brain and spinal cord (Bernard et al., 1997; Slavin et al., 1998). The relapsing-remitting course of EAE that develops in NOD/Lt mice is thought to best approximate the MS disease profile in humans.

Both NK and NKT cells have been demonstrated to regulate EAE. Mice depleted of NK cells using anti-NK1.1 mAb, develop a more severe form of EAE associated with relapse and have increased MOG-specific T cell proliferation and cytokine production (Zhang et al., 1997). Because anti-NK1.1 mAb treatment deletes both NK cells and NKT cells, $\beta 2m^{-/-}$ mice lacking class I-restricted cells including NKT cells, CD8⁺ T cells and CD4⁺CD8⁺ T cells were also investigated. Disease augmentation upon administration of anti-NK1.1 mAb also occurred in these mice, suggesting that NK cells can regulate EAE independently of T, B or NKT cells (Zhang et al., 1997). Moreover, TCR V α 14-J α 281 transgenic NOD/Lt mice that are enriched in CD1d-restricted NKT cells are protected from EAE, suggesting that NKT cells can naturally inhibit EAE (Mars et al., 2002). In addition, the therapeutic efficacy of α -GalCer (detailed in Chapter 1, Literature

Review, Section 1.2.6.1) on EAE has been recognized as NKT cell-dependent (Furlan et al., 2003).

1.6.3.1 Role of TNF superfamily molecules in EAE

TNF superfamily members play an important role in the regulation of a multitude of autoimmune diseases in humans and in animal models of autoimmune disease (detailed in Chapter 1, Literature Review, Section 1.6.1). Abnormal regulation of apoptosis and inflammation of the CNS have been implicated in EAE. Members of the TNF superfamily of receptors and ligands play an important role in apoptosis and many possess pro-inflammatory activity that might influence the severity of MS/EAE. Given the multitude of cytokines known to play a role in regulation of EAE severity (Ruuls and Sedgwick, 1998), it is plausible that some of these cytokines exert their effects by regulating the expression and function of TNF superfamily members.

1.6.3.1.1 TNF

The pro-inflammatory cytokine TNF, has long been known to play a critical role in the CNS inflammatory process associated with MS/EAE. Onset of EAE is delayed in TNF^{-/-} mice and cell movement within the CNS from the vasculature into the parenchyma is inefficient (Kassiotis and Kollias, 2001; Korner et al., 1997c; Sean Riminton et al., 1998). TNF blockade using anti-TNF antibody (Ruddle et al., 1990; Selmaj et al., 1991) or soluble forms of the p55 receptor inhibit EAE if administered during the early stages of disease (Baker et al., 1994; Korner et al., 1995; Selmaj et al., 1995; Selmaj and Raine, 1995). However, established forms of EAE are only minimally inhibited by TNF blockade, suggesting that TNF might only be critical in the initiation phase of the disease (Baker et al., 1994; Klinkert et al., 1997; Korner et al., 1997b). Moreover, TNF neutralization in progressive MS patients exacerbated disease (van Oosten et al., 1996) suggesting that TNF may play an inhibitory role in the later stages of disease.

Long-term EAE studies have revealed that although clinical symptoms are initially delayed in $\text{TNF}^{-/-}$ mice, after recovery from the acute phase of disease $\text{TNF}^{-/-}$ mice develop a chronic-progressive form of the disease, unlike WT and $\text{p75}^{-/-}$ mice that stay in remission (Kassiotis and Kollias, 2001). These studies suggest that TNF signaling through the p55 receptor of TNF, has an immunosuppressive effect that is important for the induction of tolerance during the later stages of disease. These findings might clarify the exacerbation of MS observed in progressive patients administered with TNF blocking agents. The outstanding question is whether blocking the function of the p55 TNFR in patients will inhibit inflammation yet allow the important immunosuppressive effects of TNF to occur in the later stages of disease.

1.6.3.1.2 Lymphotoxin (LT)- α

Experiments investigating the effect of LT- α on experimental models of autoimmune disease are difficult to interpret for several possible reasons. Firstly, LT- α mice have immune deficiencies such as a lack of peripheral (p)LNs and impaired humoral immune responses that are important for disease induction (De Togni et al., 1994; Koni et al., 1997), implying that differences in disease induction cannot be attributed solely to cytokine activity alone. Secondly, the LT- α gene is located within the mouse MHC (Muller et al., 1987) and backcrossing onto strains susceptible to autoimmune disease can be problematic (Steinman, 1997). Thirdly, many experimental models of autoimmune disease require the administration of pertussis toxin, which can inhibit G protein-coupled chemokine-receptor signaling and LT is known to play a role in control of chemokine levels in secondary lymphoid tissues (Cyster and Goodnow, 1995; Ngo et al., 1999).

Initial studies revealed that C57BL/6 LT- $\alpha^{-/-}$ mice were resistant to EAE induction (Sean Riminton et al., 1998), which was not surprising, given the lack of LNs and impaired humoral immunity in these mice. To address the issue of immunocompetence, BM chimeras were generated by transfer of LT- $\alpha^{-/-}$ BM into

irradiated RAG-1^{-/-} mice (Sean Riminton et al., 1998). EAE progressed normally in these mice, suggesting that LT- α does not contribute to the disease process. However, these studies were rebuked due to the use of pertussis toxin. To address this issue, Gommerman and colleagues blocked LT function in WT mice using a LT β R-Ig fusion protein and induced EAE in the absence of pertussis toxin (Gommerman et al., 2003). Administration of LT β R-Ig fusion protein inhibited MBP-induced EAE in Lewis rats and inhibited relapse of PLP-induced EAE in SJL mice. Inhibition of disease was associated with a defect in T cell responses and migration in these animals. The same researchers showed that EAE induced in the presence of pertussis toxin was not inhibited by administration of LT β R-Ig fusion protein (Gommerman et al., 2003), revealing that pertussis toxin can indeed inhibit LT- α -mediated regulation of EAE.

1.6.3.1.3 FasL

Although *lpr* and *gld* mice develop strain-dependent systemic autoimmune disease, various reports suggest that these mice are relatively resistant to MOG- and MBP-induced EAE (Malipiero et al., 1997; Sabelko et al., 1997; Waldner et al., 1997). Whilst production of Th1 cytokines and infiltration of inflammatory cells into the CNS was reported to be normal in these mice, decreased numbers of apoptotic cells were identified in CNS inflammatory lesions, implicating FasL in the tissue destruction associated with EAE (Sabelko et al., 1997; Waldner et al., 1997). In contrast, other reports suggest that EAE induction is normal in *lpr* and *gld* mice (Dittel et al., 1999; Elliott et al., 1996).

A dual role for Fas/FasL interactions in the regulation of EAE might be explained if FasL was able to induce apoptosis in target tissue and contribute to the regulation of auto-reactive T cell expansion *in vivo*. Both glial cells and MBP-reactive T cells isolated from rats express both Fas and FasL, and cell death can be induced in both populations in a Fas-dependent manner, supporting this theory (Sun et al., 1998). In addition, Fas/FasL expression is elevated in MS lesions (Bonetti and Raine, 1997; Dowling et al., 1996) and MBP-specific T cells isolated from MS patients express Fas (D'Souza et al., 1996; Pelfrey et al., 1995).

The dual role idea was most elegantly tested in adoptive transfer experiments, where MBP-specific T cells were transferred into recipient mice to induce EAE. When transferred T cells were isolated from *gld* mice, or when *lpr* recipients were used, disease was attenuated (Dittel et al., 1999; Sabelko-Downes et al., 1999). In comparison, transfer of WT T cells into *gld* recipients resulted in a prolonged and more severe form of the disease (Dittel et al., 1999; Sabelko-Downes et al., 1999). These results implicate FasL in the T cell-mediated tissue destruction associated with EAE and suggest that FasL expressed by target tissue can dampen immune responses. Evidence of a dual role for Fas/FasL in EAE is also illustrated in studies using rats administered with anti-FasL mAb. Antibody treatment at the onset of disease was protective, whereas treatment during the peak of disease prevented spontaneous recovery (Wildbaum et al., 2000). Together, these data indicate that FasL plays a dual role as both an effector in the initiation of disease and regulator during recovery from EAE. FasL can interact with Fas expressed on CNS elements causing massive tissue destruction and on infiltrating T cells helping to regulate autoreactive T cell activity.

1.7 Lymphoproliferative disorders

Homeostasis of the immune system is elegantly controlled at several stages of lymphocyte development. Autoreactive T cells are eliminated in the thymus by means of negative selection (discussed in detail in Chapter 1, Literature Review, Sections 1.5.2 and 1.5.3) (Sebzda et al., 1999) and in the periphery through excess stimulation by either foreign or self-antigens (Van Parijs and Abbas, 1998). The importance of the Fas/FasL pathway in lymphocyte homeostasis is clearly demonstrated in mice carrying loss of function mutations in the *Fas* or *FasL* genes (Adachi et al., 1993; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Watson et al., 1992) or human ALPS patients who harbor Fas/FasL deficiencies (Fisher et al., 1995; Rieux-Laucat et al., 1995) (Drappa et al., 1996).

Lymphoproliferative disease in *lpr*, *gld* and ALPS patients is uniquely characterized by an accumulation of B220⁺ DN T cells in LNs and spleen (Cohen and Eisenberg, 1991; Rieux-Laucat et al., 1999; Sneller et al., 1997). A large

number of treatments have been reported to decrease lymphoproliferative disease and the accumulation of DN T cells; neonatal thymectomy, splenectomy, chronic administration of cyclosporin A, 5-azacytidine, or SEB and chronic treatment with mAb specific for CD4, CD8, B220 or TCR V β 8 or IgM (Asensi et al., 1989; Cerny et al., 1989; de Alboran et al., 1992; Jabs et al., 1992; Kim et al., 1991; Mountz et al., 1987; Santoro et al., 1988; Steinberg et al., 1980; Yoshida et al., 1990). Some ALPS patients have been cured by allogeneic BMT (Benkerrou et al., 1997).

1.7.1 Autoimmune lymphoproliferative syndrome (ALPS)

In 1967, a condition characterized by nonmalignant lymphadenopathies associated with autoimmune features in children was described by Canale and Smith (Canale and Smith, 1967). Driven by the discovery of Fas and FasL mutations in mice, these children were later identified to have Fas pathway mutations and the condition was named ALPS or Canale-Smith syndrome (Drappa et al., 1996; Fisher et al., 1995; Rieux-Laucat et al., 1995). The ALPS condition is characterized by at least three of the following criteria: splenomegaly and/or lymphadenopathy, presence of CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ DN T cells in the blood, autoimmune features and hypergammaglobulinemia (Rieux-Laucat et al., 2003). A lymphoproliferative disease with an ALPS-like clinical pattern, but without expansion of DN T cells has also been described (Ramenghi et al., 2000). Patients diagnosed with ALPS can be subdivided into three classifications, defined functionally by the sensitivity of their lymphocytes to Fas-induced apoptosis. ALPS patients can exhibit complete Fas deficiency (ALPS 0), partial Fas deficiency (ALPS Ia and II) or absence of a Fas defect (ALPS Ib and III).

Three ALPS 0 patients have been described with homozygous *Fas* mutations. Two patients possessed mutations in the Fas DD and one possessed a stop codon in the extracellular domain of Fas leading to a complete expression defect (Kasahara et al., 1998; Rieux-Laucat et al., 1995; van der Burg et al., 2000). These patients experienced prenatal onset of the condition and exhibited very

severe lymphoproliferation accompanied by lymphocytic infiltration into the lung.

ALPS Ia is the most common form of the ALPS and is usually less severe than ALPS 0. Patients with ALPS Ia have heterozygous mutations of the *Fas* gene, which often yield truncated products or modified sequences. In most cases, onset of the condition is at 6-12 months and is characterized by defective Fas-mediated apoptosis of T and B cells, lymphadenopathy, hepato-splenomegaly and autoimmune features (Le Deist et al., 1996; Rieux-Laucat et al., 2003). Patients with ALPS II have defects in the Fas signaling pathway. Some of these patients have been shown to have mutations in caspase-10, which plays an important role in Fas mediated apoptosis (Wang et al., 1999). Mutations in the signaling molecules of other ALPS II patients are not known (Rieux-Laucat et al., 2003).

Only one case of ALPS Ib has been described. The affected patient displayed normal Fas expression and function, but a heterozygous mutation in the *FasL* gene (Wu et al., 1996). It has been argued that the phenotype of this patient does not fit the traditional criteria of classical ALPS as splenomegaly and DN T cells were not observed. ALPS III patients exhibit normal Fas/FasL-mediated apoptosis, but also exhibit a mild form of the ALPS disease (hypergammaglobulinemia, DN T cells) (Rieux-Laucat et al., 2003). It is thought that these patients may have defects in apoptotic pathways other than Fas which play a role in lymphocyte homeostasis, although this has not been demonstrated.

1.7.2 Generalized lymphoproliferative disease

Gld mice have a naturally occurring mutation in the *FasL* gene on chromosome 1 (Takahashi et al., 1994; Watson et al., 1992). This point mutation localizes to the carboxy-terminal region of the molecule and results in expression of non-functional FasL in these mice. *Gld* mice exhibit lymphadenopathy and splenomegaly (due to an accumulation of a population of nonmalignant B220⁺TCR $\alpha\beta$ ⁺CD4⁺CD8⁻ DN T cells), hypergammaglobulinemia, produce high

levels of autoantibodies and develop strain-dependent autoimmune disease (Cohen and Eisenberg, 1991; Davidson et al., 1986).

The majority of DN T cells amassing in *gld* mice derive from CD8⁺ precursors which are selected in the thymus on MHC class I molecules, the remainder express low levels of CD4 and arise independently of MHC class I (Christianson et al., 1996; Davidson et al., 1991; Giese and Davidson, 1995; Maldonado et al., 1995; Mixter et al., 1995). Total numbers of CD4⁺ and CD8⁺ T cells and B cells are also increased 10-fold in *gld* LN and are enriched for memory-type T cells and activated B cells (Budd et al., 1991; Cohen and Eisenberg, 1991; Davidson et al., 1991; Dumont et al., 1985; Giese and Davidson, 1992; Reap et al., 1996). Treatment of C3H *gld* mice with anti-CD8 mAb has been reported to prevent the accumulation of DN T cells, but not autoantibody production, suggesting that CD8⁺ T cells play a crucial role in the accumulation of DN T cells in these mice (Giese and Davidson, 1994).

C3H *gld* and BALB/c *gld* mice have been reported to develop plasmacytomas with age (Davidson et al., 1998), suggesting a role for the Fas/FasL pathway in the inhibition of tumor development. Fifty-seven percent of BALB/c *gld* and 28% of C3H *gld* mice developed plasmacytomas by twelve months of age. Tumors from these mice could be transferred and grown in severe-combined immunodeficient (scid) recipients, but were rejected in WT mice. Tumor growth was reported in the spleen and LN, and metastasized to the liver, lungs and kidney in most mice. Some tumors were also reported to grow in the ovaries and uterus. These findings support the contention that in addition to playing an important role in lymphocyte homeostasis, Fas may also play a major role in inhibiting the growth of B cell neoplasms (detailed in Chapter 1, Literature Review, Section 1.3.4.3).

1.7.3 Lymphoproliferation

Lpr mice, have a naturally occurring autosomal recessive mutation in the *Fas* gene on chromosome 19. These mice have an insertion of a retroviral transposon,

Etn, into the second intron of *Fas*, preventing normal gene transcription (Adachi et al., 1993; Watanabe-Fukunaga et al., 1992). Like *gld* mice, *lpr* mice also accumulate DN T cells in LN and spleen, develop hypergammaglobulinemia, produce high levels of autoantibodies and develop strain-dependent autoimmune disease (Cohen and Eisenberg, 1991; Davidson et al., 1986). Similarly, total numbers of CD4⁺ and CD8⁺ T cells and B cells are increased 10-fold in the LN of *lpr* mice and these cells are enriched for memory-type T cells and activated B cells (Budd et al., 1991; Cohen and Eisenberg, 1991; Davidson et al., 1991; Dumont et al., 1985; Giese and Davidson, 1992; Reap et al., 1996). Fas-deficient mice have also been generated and develop *lpr*-like disease (Senju et al., 1996). Like C3H *gld* mice, 32% of C3H *lpr* mice developed plasmacytomas by 12 months of age (Davidson et al., 1998). Moreover, there have been reports of lymphoma development in some families of ALPS patients who exhibit Fas deficiency (Drappa et al., 1996; Fisher et al., 1995; Nagata, 1997; Rieux-Laucat et al., 1995), suggesting that the Fas/FasL pathway may play a role in inhibition of tumor growth.

Lpr mice treated with anti-CD8 mAb (Giese and Davidson, 1994) and *lpr* β 2-microglobulin-deficient mice (Maldonado et al., 1995; Ohteki et al., 1995), which lack MHC class I molecules, exhibit greatly reduced numbers of DN T cells, supporting the hypothesis that DN T cells are derived from cells of the CD8 lineage and are selected on MHC class I. MRL *lpr* mice develop an autoimmune disease characterized by the development of glomerulonephritis and systemic granulomatous arteritis which is similar to systemic lupus erythematosus in humans (Andrews et al., 1978; Steinberg et al., 1984; Theofilopoulos and Dixon, 1981). Research shows that although CD8-deficient MRL *lpr* mice (Koh et al., 1995) and MRL *lpr* mice treated with anti-CD8 mAb (Merino et al., 1995) have decreased proportions of DN T cells, there is no significant effect on development of autoimmune disease. In contrast, DN T cells rapidly accumulate in CD4-deficient MRL *lpr* mice (Chesnutt et al., 1998; Koh et al., 1995) and MRL *lpr* mice treated with anti-CD4 mAb (Merino et al., 1995), however, autoimmune disease is inhibited. These data suggest that although CD8⁺ T cells are a major source of DN T cells in *lpr* mice, DN T cells do not play a role in development of

autoimmune disease in these mice. Type I interferon receptor (IFN-IR)^{-/-} *lpr* mice develop a milder form of renal disease, exhibit decreased accumulation of DN T cells in the LN and spleen and have 2-fold decreased concentrations of serum IgG compared to IFN-IR sufficient *lpr* mice (Braun et al., 2003), suggesting that type I IFNs may play a role in the regulation of autoimmune disease and lymphoaccumulation in *lpr* mice.

1.8 Project aims

Given the selective ability of rTRAIL to induce death in cancer cells, but not normal cells, tremendous excitement has been generated regarding the potential use of synthetic TRAIL alone or in combination with chemotherapy or radiotherapy for cancer patients. What has undeniably been lacking is an in depth understanding of the natural physiological role of TRAIL, the subject of which is the topic of this thesis. The aims of this study were:

- 1/ To characterize TRAIL expression on mouse leukocytes.
- 2/ To determine whether TRAIL plays a role in NK cell-mediated tumor immunity.
- 3/ To investigate the role of TRAIL and TNF in immunoregulation.
- 4/ To determine whether TRAIL plays a role in EAE.
- 5/ To investigate whether TRAIL plays a role in negative selection of thymocytes.

Increased understanding of the physiological role of TRAIL is fundamental if the TRAIL pathway is to ultimately be used safely and with efficacy in the clinic.

CHAPTER TWO

CHARACTERIZATION OF TRAIL EXPRESSION IN THE MOUSE

2.1 Abstract

Previous studies in our laboratory have shown that TRAIL is constitutively expressed on a small subset of liver NK cells in adult mice. Herein, we report that TRAIL is expressed on DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells, a surface phenotype previously reported as immature. TRAIL expression on phenotypically immature liver NK cells was decreased in IFN- γ -deficient mice, implicating IFN- γ in the regulation of constitutive TRAIL expression *in vivo*. Moreover, the majority of liver NK cells in fetal, neonatal and 2 week old mice expressed TRAIL and possessed the unique DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} phenotype, suggesting that immature NK cells predominate in the liver of newborn mice. TRAIL expression was also detected on fetal and neonatal spleen NK cells and on non-type I liver NKT cells in young mice. Fetal liver and spleen NK cells predominantly exerted TRAIL-dependent cytotoxicity, whereas liver NK cells isolated from neonatal mice, and liver MNC isolated from 2 week old mice demonstrated TRAIL-, FasL- and pfp-dependent cytotoxicity. Splenic NK cells isolated from neonatal mice demonstrated TRAIL- and pfp-dependent cytotoxicity, however TRAIL-dependent cytotoxicity had diminished by 2 weeks of age. These findings indicated that TRAIL is the key effector molecule expressed on fetal NK cells. Moreover, our data suggested that NK cells acquire the expression of Ly49 inhibitory receptors when they acquire pfp and FasL effector function. Importantly, we also describe the initial characterization of TRAIL-deficient mice generated by gene-targeting, and show that liver and spleen MNC isolated from these mice are devoid of TRAIL expression and TRAIL-mediated cytotoxicity. Analysis of TRAIL-deficient mice additionally revealed that TRAIL expression was not necessary for NK cell development. These mice will undoubtedly be of great use to further dissect the physiological and pathological roles of TRAIL.

2.2 Introduction

TRAIL is a type II transmembrane protein and member of the TNF superfamily of ligands. A number of studies using rTRAIL have demonstrated the selective ability of TRAIL to induce apoptosis in transformed and virus-infected cells, but not in most normal cells, making it particularly attractive as a potential cancer therapeutic (Ashkenazi et al., 1999; Walczak et al., 1999). However, less is known of the physiological role of TRAIL or the natural expression of TRAIL at the protein level. It was not until the development of two key research tools, a neutralizing anti-mTRAIL mAb (Kayagaki et al., 1999c) and a soluble TRAIL receptor (DR5) (Song et al., 2000), that these questions began to be addressed.

Initially, TRAIL expression was identified on human CD4⁺ T cell clones and these cells were demonstrated to mediate cytotoxicity against TRAIL-sensitive tumor cells *in vitro* (Kayagaki et al., 1999b; Thomas and Hersey, 1998). Later studies revealed a lack of TRAIL expression on freshly isolated splenic lymphocytes, thymocytes and cells isolated from LNs (Kayagaki et al., 1999c). In the same study, stimulation of T cells using anti-CD3, PHA, CD40L, lipopolysaccharide or PMA plus ionomycin did not induce TRAIL expression, although TRAIL expression could be induced on splenic NK cells after stimulation with IL-2 or IL-15. Peripheral blood T cells stimulated with type I IFNs plus anti-CD3 mAb were also identified to express TRAIL (Kayagaki et al., 1999a), as were IFN-stimulated monocytes and DCs (Fanger et al., 1999; Griffith et al., 1999). More recently, TRAIL mRNA and protein were shown to be constitutively expressed on numerous human and mouse ocular tissues, including corneal endothelium and epithelium, the iris, ciliary epithelium and neurosensory retina (Lee et al., 2002).

Recent studies in our laboratory have shown that TRAIL is constitutively expressed on a small proportion of liver NK cells in adult mice (Takeda et al., 2001). IFN- γ ^{-/-} and IFN- γ R^{-/-} mice lacked TRAIL expression, however TRAIL expression could be induced upon administration of rIFN- γ (Takeda et al., 2001).

This suggested that IFN- γ regulates constitutive TRAIL expression *in vivo* (Takeda et al., 2001).

During NK cell maturation, the cytokines that regulate specific developmental stages, and the phenotypic and functional characteristics of each developmental intermediate *in vivo*, are not clearly defined. Although a population of CD122⁺NK1.1⁻DX5⁻ committed NK cell progenitors have been identified in adult BM (Rosmaraki et al., 2001), subsequent stages of *in vivo* NK cell development, including the acquisition of cytotoxic molecules are poorly understood. A recent report by Kim and colleagues identified a high frequency of phenotypically immature NK cells in the liver of adult mice (Kim et al., 2002), raising the possibility that the liver may act as a reservoir for less differentiated NK cells. In parallel, immature CD161⁺CD56⁻ NK cells isolated from human umbilical cord blood were reported to exert TRAIL-dependent apoptosis, whereas mature CD161⁺CD56⁺ NK cells exerted pfp- and FasL-dependent cytotoxicity (Zamai et al., 1998). A similar population of immature NK cells were later identified in human PBL and demonstrated to secrete IL-5/IL-13 and to proliferate rapidly in response to IL-4 (Loza and Perussia, 2001).

Given that high proportions of immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} NK cells have been reported in the liver of adult mice (Kim et al., 2002), and immature human NK cells mediate TRAIL-dependent cytotoxicity (Loza and Perussia, 2001; Zamai et al., 1998), we hypothesized that immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells in adult mice might be the population expressing TRAIL. Indeed, herein we report that TRAIL is expressed on immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells in adult mice. Moreover, TRAIL-expressing NK cells comprised the majority of liver NK cells in fetal, neonatal and 2 week old mice. Constitutive TRAIL expression was also detected on fetal and neonatal spleen NK cells and on non-type I liver NKT cells in young mice. Our study provides evidence that TRAIL is the key effector molecule expressed by fetal and neonatal NK cells. Importantly, we also describe the initial

characterization of TRAIL-deficient mice and show that these mice are devoid of surface TRAIL expression and function.

2.3 Materials and Methods

Cell culture

The BALB/c-derived renal adenocarcinoma cell line, Renca (kindly provided by Dr. Thomas J. Sayers, National Cancer Institute, Frederick, MD, USA), the anti-mTRAIL hybridoma, N2B2 (Kayagaki et al., 1999c) (kindly provided by Dr. Hideo Yagita, Juntendo University School of Medicine, Tokyo, Japan), the BALB/c-derived mammary carcinoma cell line DA3 (kindly provided by Professor Ian McKenzie, Austin Research Institute, Heidelberg, Victoria, Australia) and the mouse YAC-1 lymphoma cell line, were maintained at 37°C and 5% carbon dioxide (CO₂) in RPMI 1640 (Invitrogen Life Technologies Corp, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) [Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia] and 2 mM L-glutamine (JRH Biosciences, Lenexa, KS, USA). The 6-thioguanine (6-TG)-resistant BALB/c-derived mammary carcinoma cell line, 4T1.2, was kindly provided by Dr. Beth Pulaski (Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD, USA) and maintained at 37°C and 5% CO₂ in α -MEM (Invitrogen Life Technologies Corp) supplemented with 10% FCS and 2 mM L-glutamine. The L929 mouse fibrosarcoma cell line was maintained at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Life Technologies Corp) supplemented with 10% (v/v) FCS and 2 mM L-glutamine.

Mice

Mice genetically deficient in TRAIL (TRAIL^{-/-}) were generated at Immunex Corporation (Seattle, WA, USA) by homologous recombination in 129-derived embryonic stem (ES) cells. Sequences between nucleotides 274-371, encoding amino acids 76-110 of the TRAIL cDNA (Wiley et al., 1995) were replaced with a cassette conferring resistance to G418. The structure of the mutation was confirmed by genomic Southern blotting and PCR analyses. Chimeras generated from TRAIL-targeted ES cells were crossed to C57BL/6 mice to achieve germ line transmission of the mutation. The resulting (C57BL/6 x 129) F₁ hybrids

were then successively backcrossed onto C57BL/6 or BALB/c backgrounds 5 generations prior to further backcrossing at Peter Mac.

Inbred BALB/c and C57BL/6 WT and BALB/c scid mice were purchased from The Walter and Eliza Hall Institute of Medical Research (WEHI) (Parkville, Victoria, Australia). The following gene-targeted mice were bred at Peter Mac: BALB/c pfp-deficient (BALB/c pfp^{-/-}) (8 back-crosses, speed mapped) (Smyth et al., 1999), BALB/c and C57BL/6 IFN- γ -deficient (IFN- γ ^{-/-}) (> 10 backcrosses) (Dalton et al., 1993), C57BL/6 IL-12-deficient (IL-12^{-/-}) (> 10 backcrosses) (Magram et al., 1996), C57BL/6 IL-18-deficient (IL-18^{-/-}) (> 10 backcrosses) (Takeda et al., 1998), C57BL/6 IL-4-deficient (IL-4^{-/-}) (> 10 backcrosses) (The Jackson Laboratory, Bar Harbor, Maine, USA), C57BL/6 TNF-deficient (TNF^{-/-}) mice (C57BL/6 ES cells) (Korner et al., 1997), C57BL/6 IFN- α -receptor1-deficient (IFN- α R1^{-/-}) (8 backcrosses) and C57BL/6 IFN- α -receptor2-deficient (IFN- α R2^{-/-}) (8 backcrosses). C57BL/6 IFN- α R1^{-/-} and C57BL/6 IFN- α R2^{-/-} mice were kindly provided by Drs. Paul Hertzog and Bernadette Scott (Center for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Clayton, Victoria, Australia), C57BL/6 TNF^{-/-} mice were obtained from Dr. Heinrich Korner (Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia). C57BL/6 RAG-2-deficient (RAG-2^{-/-}) mice (Shinkai et al., 1992) were bred at the Juntendo University School of Medicine (Bunkyo-ku, Tokyo, Japan). Adult mice 6-12 weeks of age were used in all experiments (unless otherwise stated) under specific pathogen-free conditions according to Animal Experimental Ethics Committee guidelines and approval.

Reagents

α -GalCer, a marine sponge glycolipid that activates CD1d-restricted NKT cells (Kawano et al., 1997), was provided by the Pharmaceutical Research laboratories, Kirin Brewery (Gunma, Japan) and prepared as described (Smyth et al., 2001). α -GalCer was resuspended in saline supplemented with 0.5% polysorbate-20. Concanamycin A (CMA), which inhibits pfp-mediated cytotoxicity (Takeda et al., 2001), was purchased from Wako Pure Chemicals (Osaka, Japan).

Ascites production and purification by caprylic acid

To produce anti-mTRAIL (N2B2) ascites, 6-12 week old scid mice were pretreated with 0.2 ml pristane (ICN Biomedicals Inc, Aurora, Ohio, USA) on day -10 and injected i.p. on day 0 with 2×10^7 N2B2 hybridoma cells. Mice were monitored daily for signs of abdominal swelling and ruffled fur. After a period of 1-2 weeks, mice were lightly sedated with methoxyfluorane (Medical Developments, Springvale, Victoria, Australia), and ascites fluid (1 to 1.5 ml per mouse) was drained from the peritoneum using a 22 gauge needle. After draining, the mice were monitored for any signs of shock due to fluid withdrawal such as pale eyes, ears and muzzle and breathing difficulty. Two to three days later, mice were sacrificed and all remaining ascites removed using a 22 gauge needle. At any stage, mice that showed signs of deterioration such as ruffling of hair coat, huddling, difficulty in breathing, inactivity, lethargy or loss of weight were euthanized. Tumor cells were removed from ascites by centrifugation (2500 rpm, 5 min.) and re-injected into other pristane treated scid mice. This procedure was undertaken according to Animal Experimental Ethics Committee guidelines and approval.

To purify anti-mTRAIL (N2B2) mAb from ascites, an equal volume of 60 mM sodium acetate buffer (pH 4.0) was added to the ascites and the pH of the total antibody preparation adjusted to 4.8 with 1 M sodium hydroxide. Caprylic acid (Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 1% to precipitate the majority of plasma proteins without affecting IgG. After a period of 30 min. at room temperature (RT), the antibody preparation was centrifuged (10,000 rpm, 30 min., 4°C) and the supernatant harvested. One molar Tris (pH 8.0) was added (1/10 of the total supernatant volume) to minimize acid denaturation and the antibody was dialyzed against phosphate buffered saline (PBS) overnight. Ammonium sulphate was added (0.381 g per ml of dialyzed antibody solution), the preparation stirred for a minimum of 1 h at 4°C and then centrifuged (10,000 rpm, 15 min.). The antibody-containing pellet was then resuspended in a minimum volume of PBS and re-dialyzed against PBS. After purification, the concentration of antibody was determined by spectrophotometric analysis on an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany), and

the reactivity with mTRAIL protein tested using 2PK3-mTRAIL transfectants and flow cytometric analysis.

MNC preparation

Spleen MNC were prepared by carefully placing the dissected spleen between 2 glass slides and mechanically teasing out the spleen to produce a uniform cell suspension. Spleen cells were then washed in wash buffer (1 x PBS, 2% FCS, 0.02% sodium azide) and resuspended in red blood cell lysis solution (Sigma Chemical Co.) for 5 min. to deplete red blood cells. Splenocytes were washed once more in wash buffer before use in all experiments.

To isolate liver MNC, mice were asphyxiated by CO₂ and their livers immediately perfused by injection directly into the hepatic vein with 1 x PBS. After removal of the gall bladder, the liver was cut into small pieces, passed through 200 μ m wire mesh and the cells washed 3 times in wash buffer. Cells were then resuspended in isotonic PercollTM (Amersham Biosciences, Uppsala, Sweden) (37%) and centrifuged at 693 x g for 12 min. at RT to separate lymphocytes from contaminating hepatocytes. Liver lymphocytes were then resuspended in red blood cell lysis solution for 5 min. to deplete red blood cells and washed prior to use in experiments.

Flow cytometric analysis

The following mAbs were used in multi-parameter flow cytometric analysis: anti-mouse TRAIL-phycoerythrin (PE) or -biotin (N2B2), rat IgG2a isotype control-PE or -biotin, anti-mouse CD11b-PE (M1/70), anti-mouse CD3-fluorescein isothiocyanate (FITC) or -Cy-Chrome (17A2), anti-mouse NK1.1-FITC or -biotin (PK136), anti-mouse CD49b-PE or -biotin (DX5), anti-mouse CD94-biotin (18d3), anti-mouse Ly49A-FITC (A1), anti-mouse Ly49C/I-FITC (5E6), anti-mouse Ly49D-FITC (4E5), anti-mouse Ly49G2-FITC (4D11) followed by incubation with streptavidin-PerCP, streptavidin-PE or streptavidin-allophycocyanin (APC). To avoid non-specific binding of mAbs to Fc γ R, anti-mouse CD16/32 (2.4G2) mAb was added to the mAb cocktail. Anti-mTRAIL-PE (N2B2) was obtained from e-Bioscience (San Diego, CA, USA), anti-CD8 α -PE-

Cy5.5 from Caltag Laboratories (Burlingame, CA, USA) and the remaining reagents were obtained from Becton Dickinson (BD) Biosciences Pharmingen (San Diego, CA, USA). After washing the cells with PBS/FCS/azide, cells were analyzed on a FACScan™ or FACSDiva™ (BD Biosciences Pharmingen) and the data processed by either CELLQuest™ (BD Biosciences Pharmingen) or FCS-Express 2 (De Novo Software, Thornhill, Ontario, Canada) software. Percentage of TRAIL⁺ cells was determined by gating cells that stained positive compared to isotype control staining (as depicted in Figure 2.1).

Intracellular flow cytometric analysis

The Cytofix/Cytoperm™ kit (BD Biosciences Pharmingen) was used to permeabilize liver and spleen MNC for staining of intracellular proteins. Briefly, 1×10^6 freshly isolated cells were washed twice in staining buffer (PBS/1% FCS/0.09% sodium azide), then resuspended in Cytofix/Cytoperm™ solution for 20 min. at 4°C. Cells were then washed twice in Perm/Wash™ solution, and incubated in appropriate mAb diluted in Perm/Wash™ solution for 30min. at 4°C. Cells were then washed twice in Perm/Wash™ solution, resuspended in staining buffer and analyzed by flow cytometric analysis.

Cellular cytotoxicity assay

The cytotoxic activity of spleen and liver MNC against Renca, 4T1.2, L929, DA3 and Yac-1 tumor targets was tested in a 5, 6 or 8 h ⁵¹Cr-release assays. Target cells (1×10^6) were harvested and washed once in unsupplemented RPMI 1640 (Renca, DA3, or Yac-1), DMEM (L929) or α -MEM (4T1.2). Cells were then resuspended in 100 μ l unsupplemented media containing 100 μ Ci sodium [⁵¹Cr] chromate (Amersham Biosciences) and incubated for 1 h at 37°C, 5% CO₂. Free ⁵¹Cr was removed by washing cells 3 times in unsupplemented media. Labeled target cells were then plated out in supplemented media in 96-well plates and incubated at various effector to target ratios with hepatic and splenic MNC for 5, 6 or 8 h. The assay was also performed in the presence of purified control rat IgG2a (R35-95) (10 μ g/ml), anti-mTRAIL (N2B2) mAb (10 μ g/ml), EGTA (5 mM) (Sigma Chemical Co.), anti-mFasL mAb (MFL1) (10 μ g/ml) (kindly provided by Dr. Hideo Yagita, Juntendo University School of Medicine, Tokyo,

Japan) and/or CMA (50 nM). After 5, 6 or 8 h incubation, plates were centrifuged and supernatants harvested to determine percentage ^{51}Cr release. Spontaneous ^{51}Cr release was determined by incubating target cells in medium alone and maximum release was determined by the addition of sodium dodecyl sulphate to a final concentration of 5%. Cytotoxicity was expressed as percentage specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release.

Statistical analysis

Data were analyzed using a two tailed, non-parametric, unpaired Mann Whitney test. P values < 0.05 were considered significant.

2.4 Results

2.4.1 Constitutive TRAIL expression by adult liver NK cells

Previously, our laboratory has demonstrated constitutive TRAIL expression on a proportion of DX5⁺CD3⁻ liver NK cells in adult mice (Takeda et al., 2001). The NK1.1 marker is commonly used to detect NK cells in a number of mouse strains. Although most lymphocytes that express NK1.1 also express the DX5 antigen, small subsets of DX5⁻NK1.1⁺ and DX5⁺NK1.1⁻ subsets are also found, particularly amongst the CD3⁺ population (Arase et al., 2001) (BD Biosciences Pharmingen, unpublished results). Moreover, DX5 antibody staining is frequently suboptimal for flow cytometric analysis (our own unpublished observations). Therefore, we chose to investigate TRAIL expression in the liver of adult C57BL/6 WT mice using antibodies to NK1.1 and CD3. Constitutive TRAIL expression was detected on a proportion of freshly isolated liver NK1.1⁺CD3⁻ cells ($18.5 \pm 4.5\%$), but not on NK1.1⁻CD3⁺, NK1.1⁺CD3⁺ or NK1.1⁻CD3⁻ cells (Figure 2.1). Using antibodies to NK1.1 and CD3, we also showed that TRAIL is not expressed on freshly isolated spleen or BM NK1.1⁺CD3⁻, NK1.1⁻CD3⁺, NK1.1⁺CD3⁺ or NK1.1⁻CD3⁻ cells in adult C57BL/6 WT mice (Figure 2.2).

2.4.2 IFN- γ regulates constitutive TRAIL expression *in vivo*

To ascertain whether cytokines regulate TRAIL expression on liver NK cells, constitutive TRAIL expression was evaluated in various cytokine-deficient mice. Although Takeda and colleagues had previously reported that adult IFN- γ -deficient mice are devoid of liver NK cell TRAIL expression (Takeda et al., 2001), we detected TRAIL expression on a minor proportion ($5.5 \pm 4.5\%$) of liver NK cells in adult IFN- γ -deficient mice (Figure 2.3). Nonetheless, proportions of TRAIL⁺ hepatic NK cells in adult C57BL/6 IFN- γ ^{-/-} mice were significantly decreased compared to C57BL/6 WT mice ($18.5 \pm 4.5\%$) ($P < 0.05$, Figure 2.3). TRAIL expression was additionally evaluated in IL-12^{-/-}, IL-18^{-/-}, IL12 IL18^{-/-}, IL-4^{-/-}, TNF^{-/-}, IFN α R1^{-/-} and IFN α R2^{-/-} mice, although deficiency of these cytokines/cytokine receptors had no significant effect on adult liver NK cell TRAIL expression (Appendix I). Together, these data suggest that

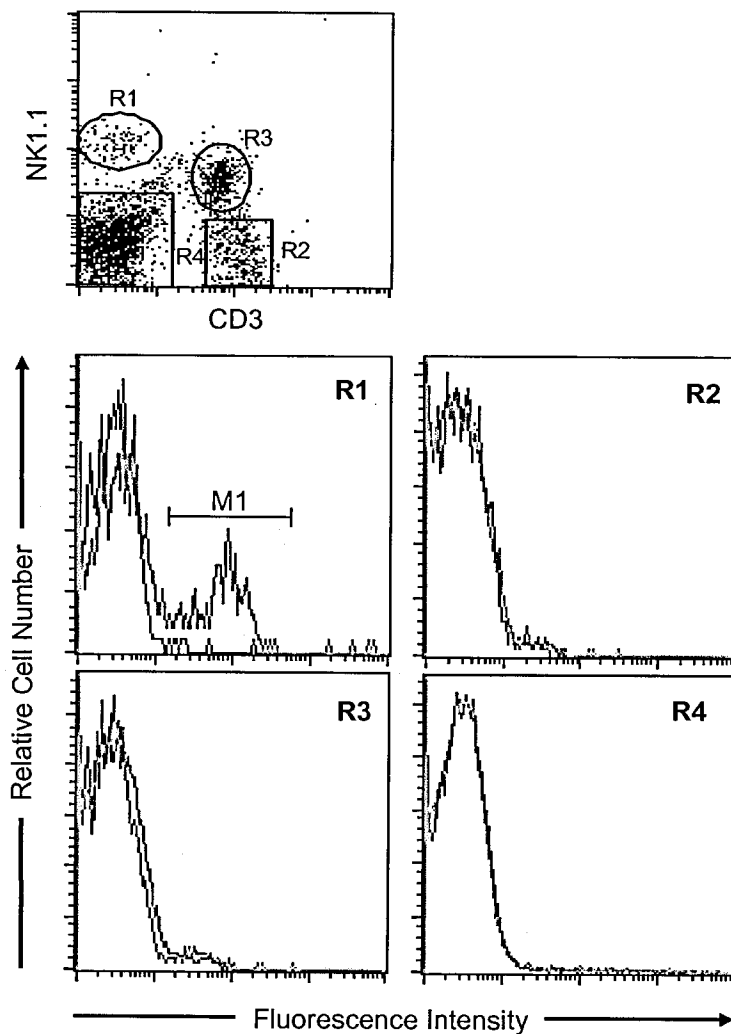


Figure 2.1. Constitutive TRAIL expression by mouse liver NK cells. Liver MNC isolated from adult C57BL/6 mice were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. The R1 gate comprises NK1.1⁺CD3⁻ cells, R2 comprises NK1.1⁻CD3⁺ cells, R3 comprises NK1.1⁺CD3⁺ cells and R4 comprises NK1.1⁻CD3⁻ cells. Histograms demonstrate TRAIL/isotype control antibody staining on the gated MNC populations (R1-R4). Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Percentage of TRAIL⁺ cells was determined by gating cells (see gate M1) that stained positive compared to isotype control. Data are representative of over 20 independent experiments.

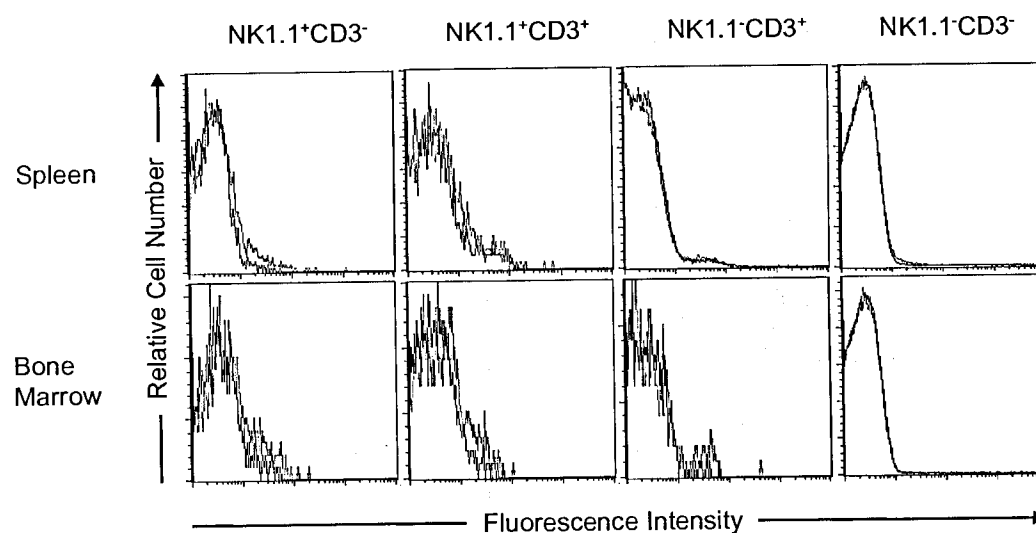


Figure 2.2. Absence of constitutive TRAIL expression by spleen and bone marrow (BM) mononuclear cells (MNC). Splenocytes and BM MNC isolated from adult C57BL/6 mice were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on NK1.1⁺CD3⁻, NK1.1⁺CD3⁺, NK1.1⁻CD3⁺ and NK1.1⁻CD3⁻ cells isolated from the spleen and BM. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Data are representative of 6 independent experiments for spleen and 2 for BM.

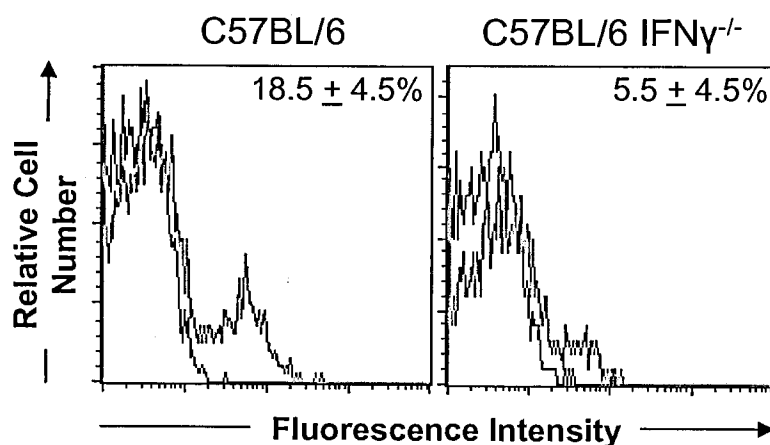


Figure 2.3. IFN- γ regulates constitutive TRAIL expression in vivo. Liver MNC isolated from adult C57BL/6 and C57BL/6 IFN- $\gamma^{-/-}$ mice were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control staining on gated NK1.1 $^{+}$ CD3 $^{+}$ cells. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Percentage of TRAIL $^{+}$ cells was determined by gating cells that stained positive over isotype control staining. Data are representative of the analysis of 20 C57BL/6 and 10 C57BL/6 IFN- $\gamma^{-/-}$ mice. Numbers represent mean \pm standard error of the mean (SEM) of percentage TRAIL $^{+}$ cells.

endogenously produced IFN- γ is an important maintenance factor for constitutive TRAIL expression in the liver.

2.4.3 Surface phenotype of TRAIL-expressing adult liver NK cells

We next chose to investigate whether TRAIL⁺ liver NK cells in adult C57BL/6 WT mice displayed an immature phenotype as previously defined by Kim and colleagues (Kim et al., 2002). DX5, CD11b, Ly49 and CD94 staining was compared on electronically gated CD3⁻NK1.1⁺ liver NK cells, TRAIL⁻ liver NK cells and TRAIL⁺ liver NK cells. The majority of TRAIL⁻ liver NK cells were DX5^{hi} and CD11b^{hi}, whereas the majority of TRAIL⁺ NK cells were DX5^{lo} and CD11b^{lo} (Figure 2.4). Whilst $68.2 \pm 10.2\%$ of whole liver NK cells expressed Ly49s (a cocktail of antibodies to Ly49A, C/I, D, and G2 was used), the vast majority ($92.4 \pm 8.5\%$) of TRAIL⁺ NK cells were Ly49⁻ (Figure 2.4). Moreover, whole hepatic NK cells were either CD94^{hi} or CD94^{lo}, whilst TRAIL⁺ NK cells were predominantly CD94^{hi} ($88.4 \pm 9.6\%$) (Figure 2.4). These data indicated that TRAIL is expressed on DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells, a phenotype previously reported for immature NK cells (Kim et al., 2002).

2.4.4 IFN- γ regulates NK cell maturation *in vivo*

Since we had shown that TRAIL was expressed on phenotypically immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} NK cells (Figure 2.4), we subsequently evaluated proportions of immature NK cells in C57BL/6 IFN- γ ^{-/-} mice. As expected, we observed decreased TRAIL expression on phenotypically immature liver NK cells in C57BL/6 IFN- γ ^{-/-} mice compared to C57BL/6 WT mice (Figure 2.5). In addition to a defect in TRAIL expression, proportions of phenotypically immature DX5^{lo}CD94^{hi}, CD11b^{lo}Ly49⁻ and DX5^{lo}Ly49⁻ cells were decreased in C57BL/6 IFN- γ ^{-/-} mice compared to C57BL/6 WT mice (Figure 2.5). Together, these data suggest that IFN- γ may play a minor role in the regulation of NK cell maturation *in vivo*.

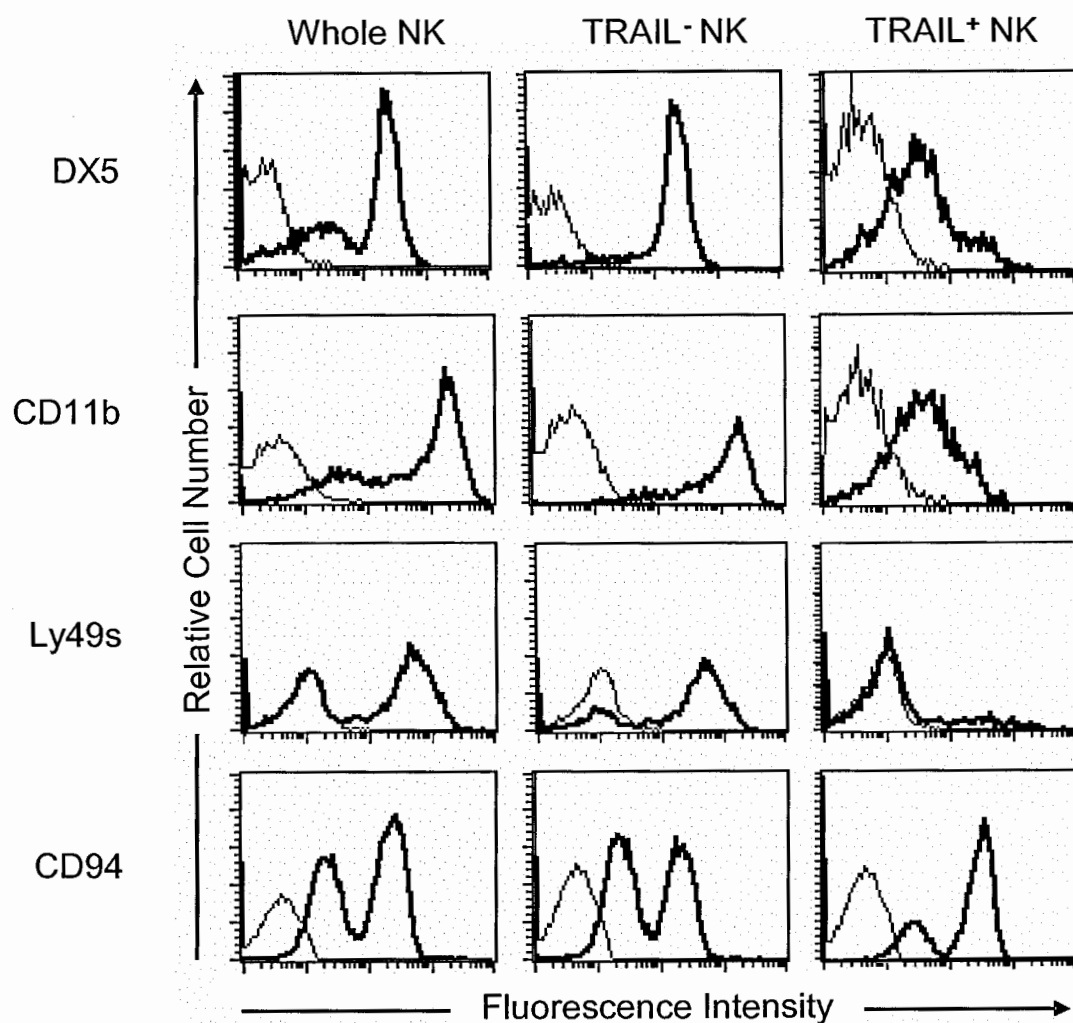


Figure 2.4. TRAIL expression by DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells. Liver MNC isolated from adult C57BL/6 mice were stained with combinations of antibodies to CD3, NK1.1, DX5, CD11b, Ly49s (A, C/I, G2, D), CD94, TRAIL or isotype control mAb. Histograms represent DX5, CD11b, Ly49s and CD94 staining (bold lines) or isotype control staining (thin lines) on whole liver NK cells (NK1.1⁺CD3⁺), TRAIL⁻ liver NK cells and TRAIL⁺ liver NK cells. Percentage of positively stained cells was determined by gating cells that stained positive compared to isotype control staining. Data are representative of 2 independent experiments.

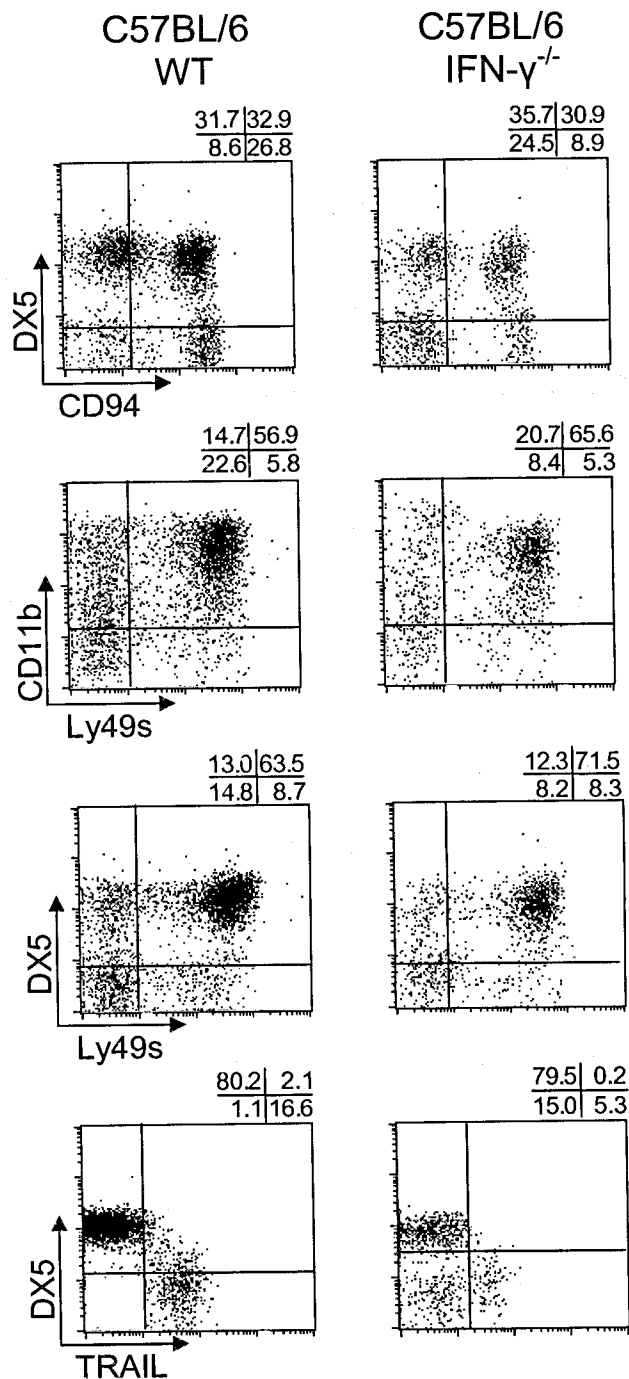


Figure 2.5. NK cell subpopulations in the liver of IFN- γ ^{-/-} mice. Liver MNC isolated from adult C57BL/6 WT and C57BL/6 IFN- γ ^{-/-} mice were stained with combinations of antibodies to CD3, NK1.1, DX5, CD94, CD11b, Ly49s (A, C/I, G2, D) and TRAIL. Representative CD94 versus DX5, Ly49s versus CD11b, Ly49s versus DX5 and TRAIL versus DX5 are shown for gated NK1.1⁺CD3⁻ cells. Numbers represent the percentages of cells in each quadrant. Data are representative of 3 independent experiments.

2.4.5 Inter-strain comparison of TRAIL expression by NK cells

We next compared constitutive TRAIL expression in the liver of adult C57BL/6 WT and BALB/c WT mice. As NK1.1 is not expressed in BALB/c WT mice, antibodies to IL-2 receptor β chain (IL-2R β) (CD122) and CD3 were used to investigate TRAIL expression in the liver. IL-2R β is expressed on all NK cells, a subset of CD8⁺ spleen T cells, and a small population of resting B cells. Moreover, early NK cell precursors express the IL-2R β chain before acquiring NK1.1 expression (Ikawa et al., 1999; Rosmaraki et al., 2001; Williams et al., 2000; Williams et al., 1997). Constitutive TRAIL expression was demonstrated on a proportion of freshly isolated liver IL-2R β ⁺CD3⁻ NK cells, but not on IL-2R β ⁻CD3⁺, IL-2R β ⁺CD3⁺ or IL-2R β ⁻CD3⁻ cells in C57BL/6 WT and BALB/c WT mice (Figure 2.6). Although proportions and absolute cell numbers of IL-2R β ⁺CD3⁻ liver NK cells were equivalent in both strains of mice, proportions of TRAIL⁺IL-2R β ⁺CD3⁻ liver NK cells were significantly higher in C57BL/6 WT ($36.6 \pm 0.9\%$) compared to BALB/c WT ($7.5 \pm 1.2\%$) mice ($P < 0.05$, Figure 2.6). Phenotypic analysis confirmed that TRAIL expression was restricted to DX5⁺IL-2R β ⁺CD3⁻ NK cells in C57BL/6 WT and BALB/c WT mice (Figure 2.7). TRAIL was expressed on a higher proportion of DX5⁺IL-2R β ⁺CD3⁻ NK cells in C57BL/6 WT ($63.5 \pm 0.5\%$) compared to BALB/c WT ($26.9 \pm 2.2\%$) mice ($P < 0.05$, Figure 2.7). These data suggest that constitutive TRAIL expression detected on IL-2R β ⁺CD3⁻ liver NK cells is higher in C57BL/6 WT compared to BALB/c WT mice.

2.4.6 Increased TRAIL expression by NK cells in young mice

Given that TRAIL⁺ liver NK cells expressed a surface phenotype previously described as "immature", we examined whether such NK cells were prevalent in the early stages of immune system development in fetal and neonatal mice. Fetal and neonatal splenic NK cells are devoid of Ly49A/C/G2/D/ and I expression, although Ly49 expression gradually increases from 1 week of age and reaches adult levels by 6-8 weeks of age (Dorfman and Raulet, 1998; Salcedo et al., 2000; Sivakumar et al., 1997). In contrast, Ly49E is highly expressed on fetal NK cells, but decreases dramatically after birth (Van Beneden et al., 2001). In addition,

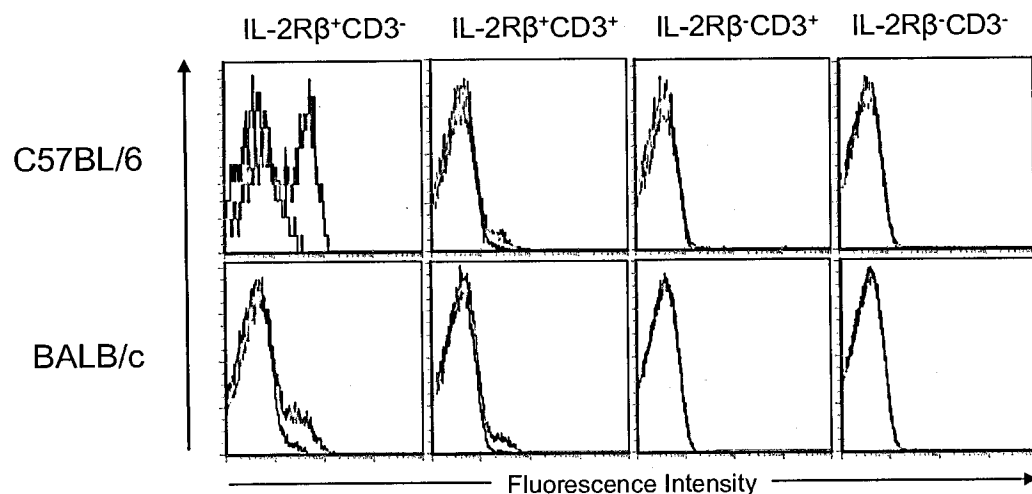


Figure 2.6. Comparative TRAIL expression by C57BL/6 and BALB/c liver NK cells. Liver MNC isolated from adult C57BL/6 or BALB/c mice were stained with antibodies to CD3, IL-2R β and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on IL-2R β ⁺CD3⁻, IL-2R β ⁺CD3⁺, IL-2R β ⁻CD3⁺ and IL-2R β ⁻CD3⁻ cells within the liver. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Percentage of TRAIL⁺ cells was determined by gating cells that stained positive compared to isotype control staining. Data are representative of 2 independent experiments.

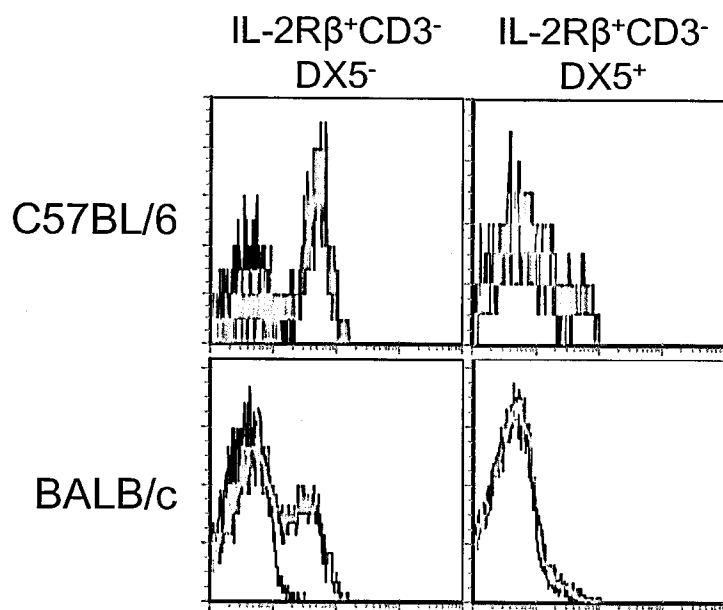


Figure 2.7. TRAIL expression by IL-2R β ⁺CD3⁻DX5⁻ cells within the liver. Liver MNC isolated from adult C57BL/6 or BALB/c mice were stained with antibodies to CD3, IL-2R β , DX5 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on IL-2R β ⁺CD3⁻DX5⁻ and IL-2R β ⁺CD3⁻DX5⁺ cells within the liver. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Percentage of TRAIL⁺ cells was determined by gating cells that stained positive compared to isotype control staining. Data are representative of 2 independent experiments.

~90% of fetal splenic NK cells compared to ~50% of adult splenic NK cells express high levels of CD94 (Sivakumar et al., 1999; Van Beneden et al., 2001). As we had shown that TRAIL⁺ liver NK cells in adult mice are CD94^{hi} and lack Ly49A/C/G2/D/I expression (Figure 2.4), we examined TRAIL expression on fetal liver NK cells or liver NK cells isolated from young mice. A low level of constitutive TRAIL expression were observed on the majority of fetal liver NK cells, whilst high levels of TRAIL expression were observed on the majority of neonatal liver NK cells (Figure 2.8). Moreover, TRAIL⁺ fetal and neonatal liver NK cells were DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi}, similar to TRAIL⁺ adult liver NK cells (personal communication, Dr. Kazuyoshi Takeda). At 2 weeks of age, 85.5 ± 9.3% of liver NK cells were TRAIL⁺, compared to 62.5 ± 10.5% at 4 weeks of age and 27.4 ± 9.1% at 8 weeks of age (Figure 2.8). Like fetal and newborn TRAIL-expressing liver NK cells, TRAIL⁺ liver NK cells in 2 week old mice were DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} (Figure 2.9). These data demonstrated that immature TRAIL-expressing DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells were predominant in fetal and neonatal mouse liver, but decreased in proportion with age.

To determine whether the liver microenvironment is important for the increased TRAIL expression on NK cells in fetal/neonatal life, TRAIL expression was additionally examined on fetal and neonatal splenic NK cells, and splenic NK cells isolated from young mice. In contrast to adult spleen NK cells, constitutive TRAIL expression was observed on the majority of fetal and neonatal spleen NK cells (Figure 2.8), but interestingly, at a lower level compared to TRAIL expression on fetal and neonatal liver NK cells (Figure 2.8). By 2 weeks of age, flow cytometric analysis revealed minimal TRAIL staining, and from 4 weeks of age TRAIL was not detected in the spleen (Figure 2.8). Similar to TRAIL⁺ liver NK cells, TRAIL⁺ fetal and neonatal splenic NK cells were DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi}, although these cells rapidly disappeared with the appearance of DX5^{hi}CD11b^{hi}Ly49⁺CD94^{lo} NK cells (personal communication, Dr Kazuyoshi Takeda). Even at 2 weeks of age, when minimal TRAIL staining was detected on spleen NK cells, increased proportions of DX5^{lo}, CD11b^{lo} and Ly49^{lo/-} NK cells

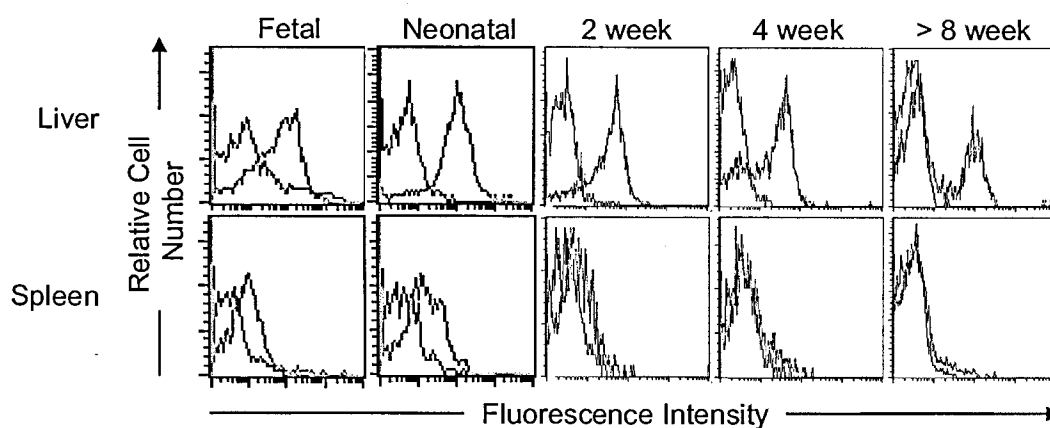


Figure 2.8. Increased constitutive TRAIL expression in the liver and spleen of young mice. Liver and spleen MNC isolated from fetal (embryo day 17), neonatal, and 2, 4 and 8 week old C57BL/6 mice were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on NK1.1⁺CD3⁺ cells. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Percentage of TRAIL⁺ cells was determined by gating cells that stained positive compared to isotype control staining. Data are representative of the analysis of 4 mice at each time point.

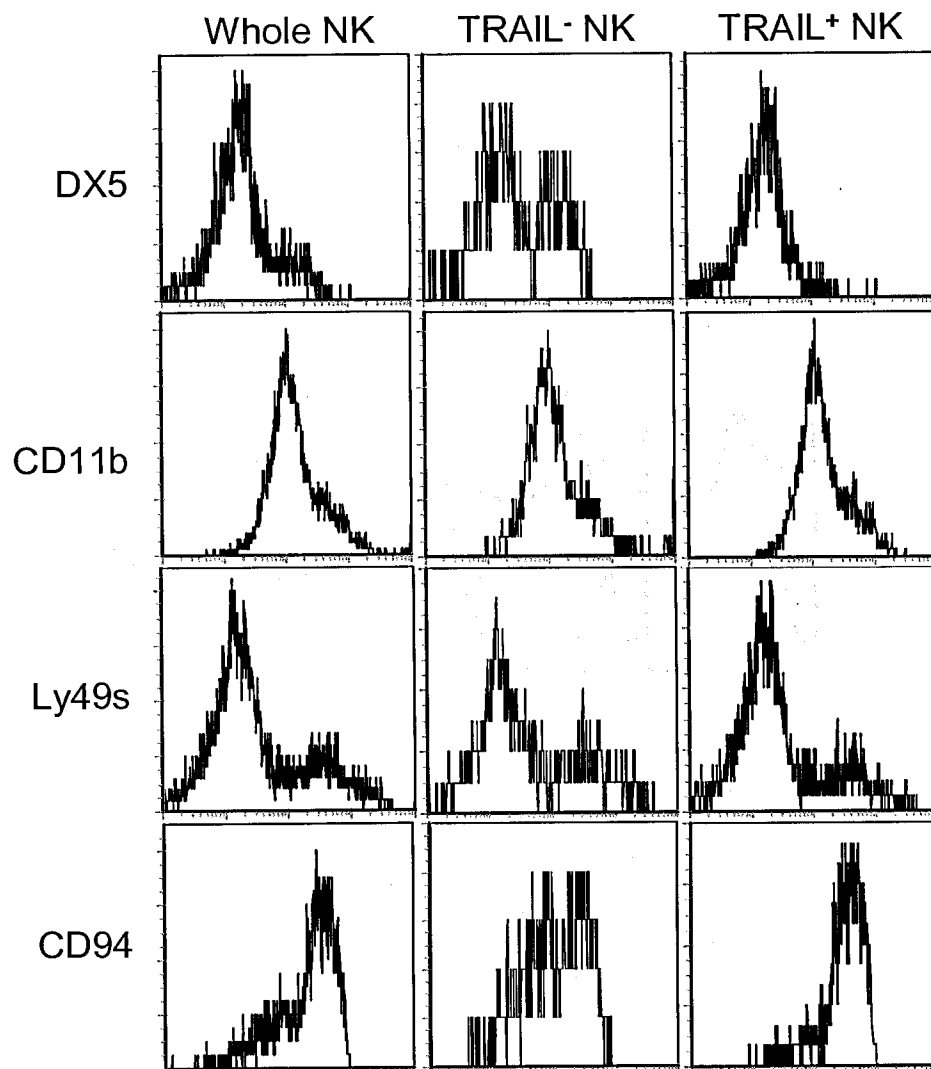


Figure 2.9. Increased proportions of DX5^{lo}CD11b^{lo}Ly49⁺CD94^{hi} liver NK cells in young mice. Liver MNC isolated from 2 week old C57BL/6 mice were stained with combinations of antibodies to CD3, NK1.1, DX5, CD11b, Ly49s (A, C/I, G2, D), CD94, TRAIL or isotype control mAb. Histograms represent DX5, CD11b, Ly49s and CD94 staining on whole NK cells, TRAIL⁻ NK cells and TRAIL⁺ NK cells. Data are representative of 2 independent experiments.

were observed in the spleen compared to adult mice (Figure 2.10). This suggested that immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} spleen NK cells express TRAIL, although these cells rapidly decreased in proportion after birth.

Given that IFN- γ had previously been implicated in the regulation of constitutive TRAIL expression *in vivo* (Takeda et al., 2001), we assessed liver NK cell TRAIL expression in C57BL/6 IFN- γ ^{-/-} mice at 2, 4 and 8 weeks of age. Proportions of TRAIL⁺ liver NK cells in 2 week old C57BL/6 IFN- γ ^{-/-} mice (45.4 \pm 1.4%) and 4 week old C57BL/6 IFN- γ ^{-/-} mice (47.3 \pm 2.4%) were significantly elevated compared to proportions of these cells in adult C57BL/6 IFN- γ ^{-/-} mice (5.5 \pm 4.5%) ($P < 0.03$), but were decreased compared to C57BL/6 WT mice of the same age (Figure 2.11). These data confirm a requirement for IFN- γ in the maintenance of constitutive TRAIL expression on liver NK cells during adult life. However, IFN- γ is not absolutely required for constitutive TRAIL expression *in vivo*.

2.4.7 TRAIL expression by non-type I natural killer T (NKT) cells in young mice

To date, the only lymphocyte populations reported to constitutively express TRAIL are a proportion of liver NK cells in adult mice (Takeda et al., 2001) (Figure 2.1), and fetal/neonatal liver and spleen NK cells reported in the present study (Figure 2.8). As TRAIL expression is increased on NK cells isolated from fetal, neonatal and young mice, we examined whether TRAIL is expressed on other liver and spleen MNC populations in 2, 4 and 8 week old C57BL/6 WT mice. Our study determined that TRAIL was not expressed on NK1.1⁻CD3⁺ or NK1.1⁻CD3⁻ cells in the spleen or liver of 2, 4 or 8 week old mice, nor was TRAIL expressed on NK1.1⁺CD3⁺ cells in the spleen (data not shown). In contrast, TRAIL expression was detected on liver NK1.1^{hi}CD3^{hi} cells in 2 week old C57BL/6 mice (Figure 2.12A), although NK1.1^{lo}CD3^{lo} cells in 4 and 8 week old C57BL/6 mice lacked TRAIL expression (Figure 2.12A). TRAIL expression was also assessed on NK1.1⁺CD3⁺ cells isolated from 2, 4 and 8 week old

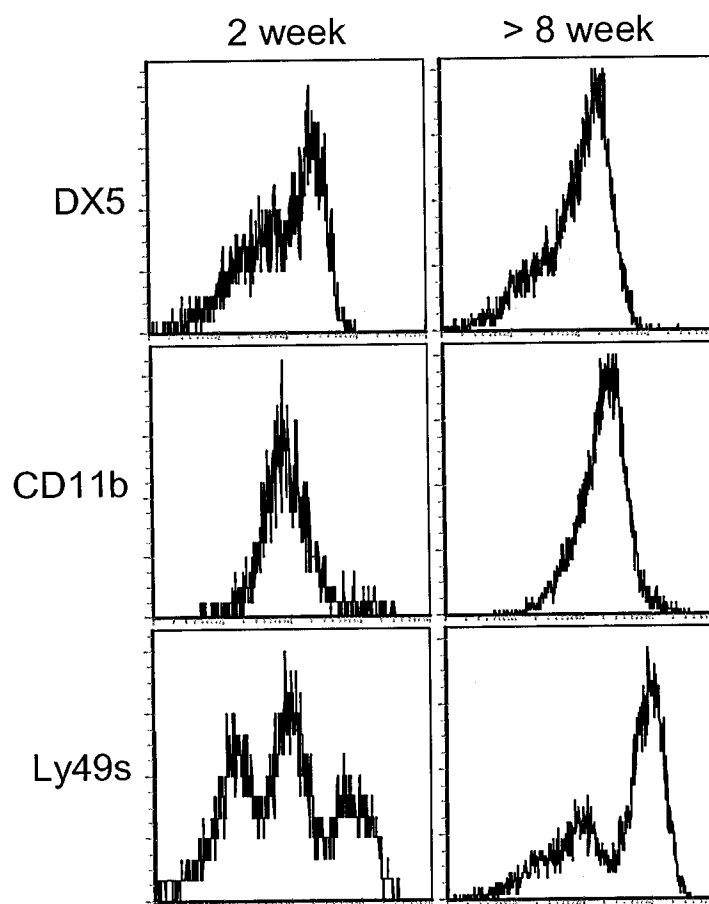


Figure 2.10. Increased proportions of DX5^{lo}CD11b^{lo}Ly49⁺ spleen NK cells in young mice. Spleen MNC isolated from C57BL/6 2 or >8 weeks of age were stained with combinations of antibodies to CD3, NK1.1, DX5, CD11b, Ly49s (A, C/I, G2, D), TRAIL or isotype control mAb. Histograms represent DX5, CD11b, and Ly49s staining on whole spleen NK cells isolated from 2 and 8 week old mice. Data are representative of 2 independent experiments.

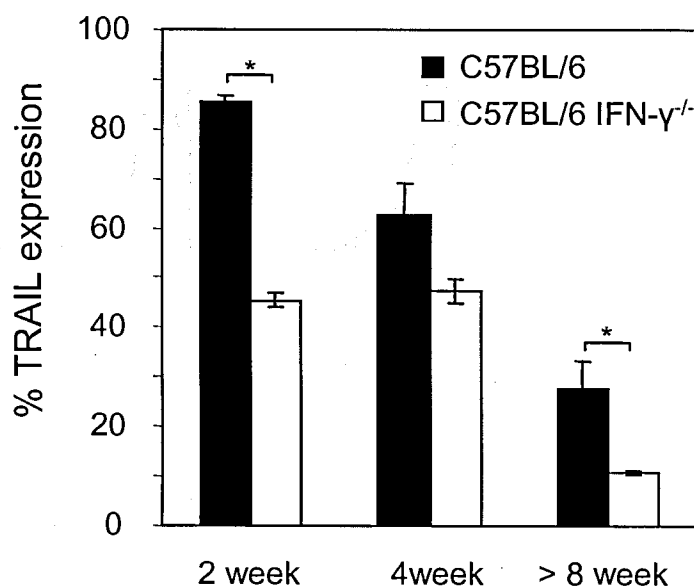


Figure 2.11. Decreased constitutive TRAIL expression in the liver of IFN- γ -deficient mice. Liver MNC isolated from C57BL/6 WT or C57BL/6 IFN- $\gamma^{-/-}$ mice 2, 4 or > 8 weeks of age were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Percentage TRAIL expression on liver NK1.1⁺CD3⁻ cells from C57BL/6 or C57BL/6 IFN- $\gamma^{-/-}$ mice is shown at 2, 4 and >8 weeks of age. Data represent mean \pm SEM for 4 C57BL/6 and 4 C57BL/6 IFN- γ -deficient mice at 2 and 4 weeks of age, and 8 C57BL/6 and 8 C57BL/6 IFN- γ -deficient mice at 8 weeks of age. Asterisks indicate the groups if mice that are significantly different (* $P < 0.03$).

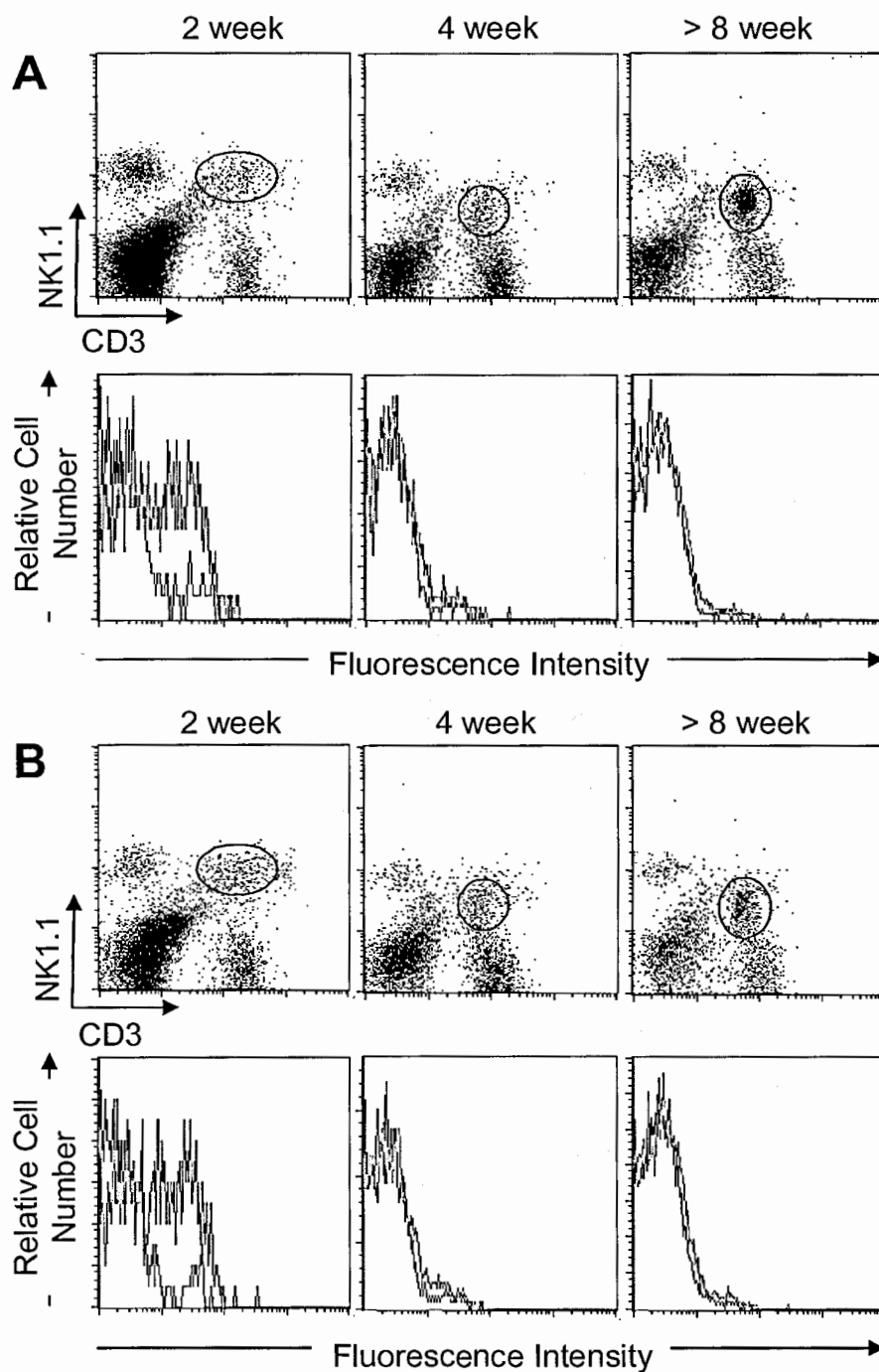


Figure 2.12. Constitutive TRAIL expression by liver NK1.1⁺CD3⁺ cells in young mice. Liver MNC isolated from C57BL/6 or C57BL/6 IFN- γ ^{-/-} mice 2, 4 or >8 weeks of age were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Representative CD3 versus NK1.1 FACS profiles and histograms demonstrating TRAIL/isotype control antibody staining on gated NK1.1⁺CD3⁺ cells from (A) C57BL/6 mice or (B) C57BL/6 IFN- γ ^{-/-} mice at 2, 4 and > 8 weeks of age are shown. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Gated NK1.1⁺CD3⁺ cells are shown on the FACS profiles. Data are representative of the analysis of a minimum of 4 C57BL/6 and 4 C57BL/6 IFN- γ ^{-/-} mice at each time point.

C57BL/6 IFN- γ ^{-/-} mice (Figure 2.12B). Equivalent proportions of TRAIL-expressing NK1.1^{hi}CD3^{hi} cells were detected in 2 week old C57BL/6 IFN- γ -deficient mice (Figure 2.12B) compared to C57BL/6 WT mice at the same age (Figure 2.12A). This suggested that IFN- γ was not critical for the regulation of constitutive TRAIL expression on mouse NK1.1^{hi}CD3^{hi} cells.

Although NK1.1 is considered a reliable marker for identification of NKT cells, some conventional CD8 α β ⁺ T cells can acquire NK1.1 expression upon activation (Emoto and Kaufmann, 2003). The vast majority of activated CD8⁺ T cells that acquire NK1.1 expression co-express CD8 α and CD8 β , whereas most CD8⁺ NKT cells express CD8 α but not CD8 β . To ascertain whether TRAIL-expressing NK1.1^{hi}CD3^{hi} cells in 2 week old mice were NKT cells or activated T cells that had acquired NK1.1 expression, CD8 α and CD8 β expression was examined on these cell populations. A small proportion of NK1.1^{hi}CD3^{hi} cells in 2 week old mice expressed CD4 (4.6 ± 0.5 %), negligible NK1.1^{hi}CD3^{hi} cells expressed CD8 β and 51.4 ± 1.3 % expressed CD8 α (Figure 2.13). The lack of CD8 β staining on NK1.1^{hi}CD3^{hi} cells in 2 week old mice suggested that these cells are NKT cells, rather than activated CD8⁺ T cells that have acquired NK1.1 expression.

Classical (type I) NKT cells are CD4⁺ or CD4⁻CD8⁻, express the invariant V α 14-J α 18 TCR, are restricted by CD1d, and are most abundant in the liver (Emoto et al., 1995; Ohteki and MacDonald, 1994; Godfrey et al., 2004). Although the natural ligand(s) for classical (type I) NKT cells are unknown, α -GalCer isolated from a marine sponge can act as a specific CD1d-restricted ligand for these cells (Kawano et al., 1997). We therefore examined whether TRAIL is expressed on classical NKT cells in the liver of 2 week old mice using α -GalCer-loaded CD1d tetramer to specifically identify these cells. α -GalCer tetramer bound to 1.9 ± 0.3 % of total liver MNC from 2 week old mice, whilst control unloaded tetramer did not bind (Figure 2.14A). Moreover, TRAIL was not detected on cells that

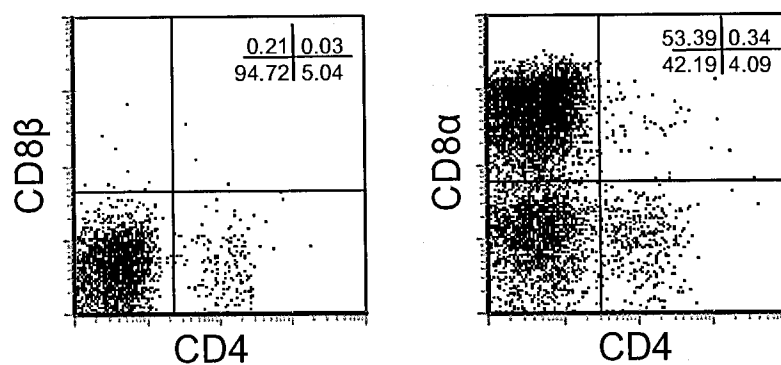


Figure 2.13. Liver NK1.1⁺CD3⁺ cells in young mice express CD8α but not CD8β. Liver MNC isolated from C57BL/6 mice 2 weeks of age were stained with antibodies to CD3, NK1.1, CD4 and CD8α or CD8β. CD4 versus CD8β and CD4 versus CD8α FACS profiles are shown for NK1.1⁺CD3⁺ cells.

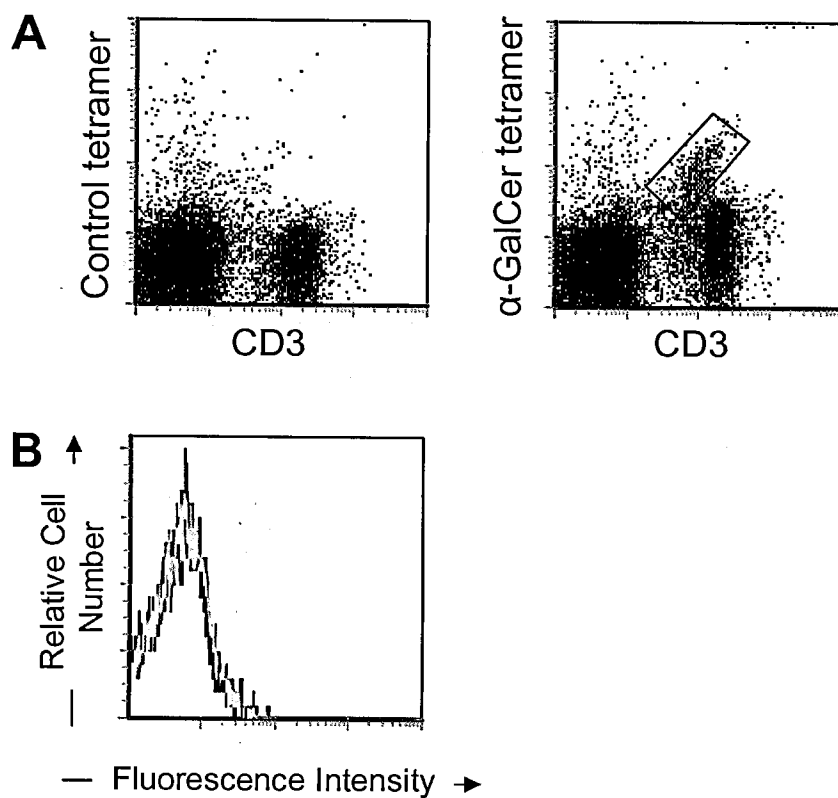


Figure 2.14. Classical (type I) NKT cells in young mice do not express TRAIL. Liver MNC isolated from C57BL/6 mice 2 weeks of age were stained with α -GalCer-loaded or control unloaded CD1d tetramer and CD3, TRAIL or isotype control mAb. (A) Representative CD3 versus control tetramer or CD3 versus α -GalCer tetramer staining on liver MNC, gate indicates tetramer positive cells. (B) Histogram demonstrating TRAIL (red) and isotype control mAb (black) staining on the gated α -GalCer tetramer positive classical (type I) NKT cell population. Results are representative of 3 independent experiments.

bound α -GalCer tetramer (Figure 2.14B), suggesting that TRAIL is not expressed on classical (type I) NKT cells.

In addition to classical (type I) NKT cells, non-type I NKT cells that express a diverse TCR and do not bind α GalCer also exist. Non-type I NKT cells are divided into TCR α/β and TCR γ/δ subsets and are either CD8⁺ or CD4⁻CD8⁻ (Emoto et al., 2001; Emoto et al., 2000). Both subsets of non-type I NKT cells are found in the liver and are particularly abundant during the first two weeks of life, with TCR γ/δ NKT cells increasing in number before TCR α/β NKT cells (Emoto et al., 2001; Emoto et al., 2000). We hypothesized that TRAIL might be expressed on non-type I NKT cells that represent a substantial NKT cell subset early in life, but decrease in proportion with age. Flow cytometric analysis revealed that ~2-3% of total liver MNC in 2 week old mice were non-type I TCR α/β NKT cells, and a similar proportion were non-type I TCR γ/δ NKT cells (Figure 2.15). Importantly, TRAIL was expressed on TCR α/β (Figure 2.15A) and TCR γ/δ non-type I NKT cells (Figure 2.15B). Moreover, TRAIL was expressed on both CD8⁺ and CD4⁻CD8⁻ subsets of these cells (Figure 2.15). These findings provided evidence that TRAIL is constitutively expressed on non-type I NKT cells in young mice.

2.4.8 Cytotoxic activity of liver NK cells isolated from young mice

As we had shown that TRAIL is expressed on fetal/neonatal liver and spleen NK cells (Figure 2.8), we then examined whether TRAIL was functional on these cells. Initially, the cytotoxicity of liver and spleen NK cells isolated from fetal and neonatal C57BL/6 RAG-2^{-/-} mice was tested against TRAIL-, FasL- and pfp-sensitive L929 target cells. Fetal liver and spleen NK cells predominantly exerted TRAIL-dependent cytotoxicity (Figure 2.16A and B). In contrast, neonatal liver NK cells exerted TRAIL-, FasL-, and pfp-dependent cytotoxicity (Figure 2.17A), whilst neonatal spleen NK cells demonstrated TRAIL- and pfp-dependent cytotoxicity, but did not exert FasL-dependent cytotoxicity (Figure 2.17B). These data suggested that TRAIL is the only cytotoxic effector molecule expressed by

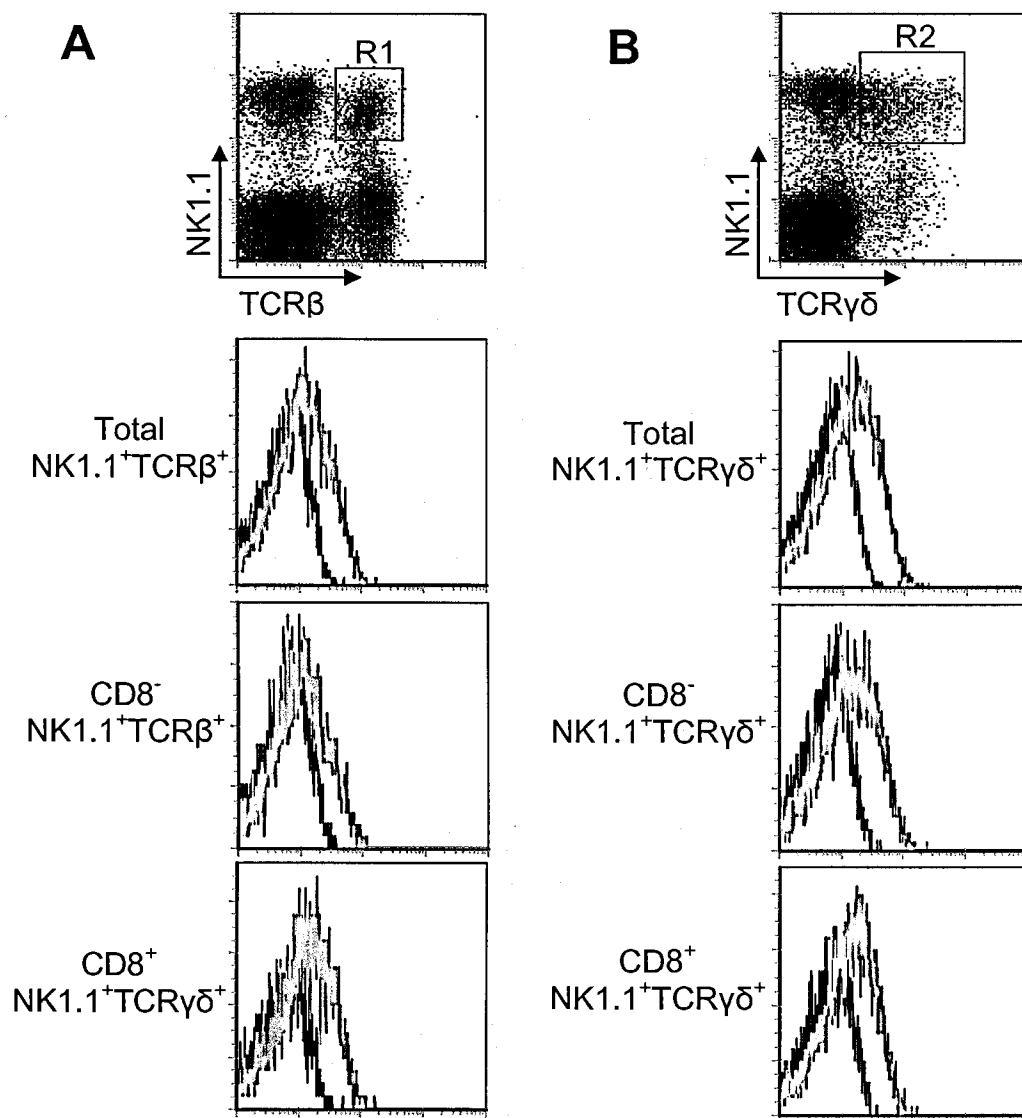


Figure 2.15. Constitutive TRAIL expression by non-type I liver NKT cells in young mice. Liver MNC isolated from C57BL/6 mice 2 weeks of age were stained with antibodies to TCR β , TCR $\gamma\delta$, NK1.1, CD8 and TRAIL or isotype control mAb. **(A)** TCR β versus NK1.1 FACS profile, and histograms demonstrating TRAIL/isotype control antibody staining on total NK1.1⁺TCR β ⁺ (R1), NK1.1⁺TCR β ⁺CD8⁻ or NK1.1⁺TCR β ⁺CD8⁺ cells, **(B)** TCR $\gamma\delta$ versus NK1.1 FACS profile, and histograms demonstrating TRAIL/isotype control antibody staining on total NK1.1⁺TCR $\gamma\delta$ ⁺ (R2), NK1.1⁺TCR $\gamma\delta$ ⁺CD8⁻ or NK1.1⁺TCR $\gamma\delta$ ⁺CD8⁺ cells. Red lines represent staining with anti-TRAIL mAb and black lines represent staining with isotype matched control mAb. Results are representative of 2 independent experiments

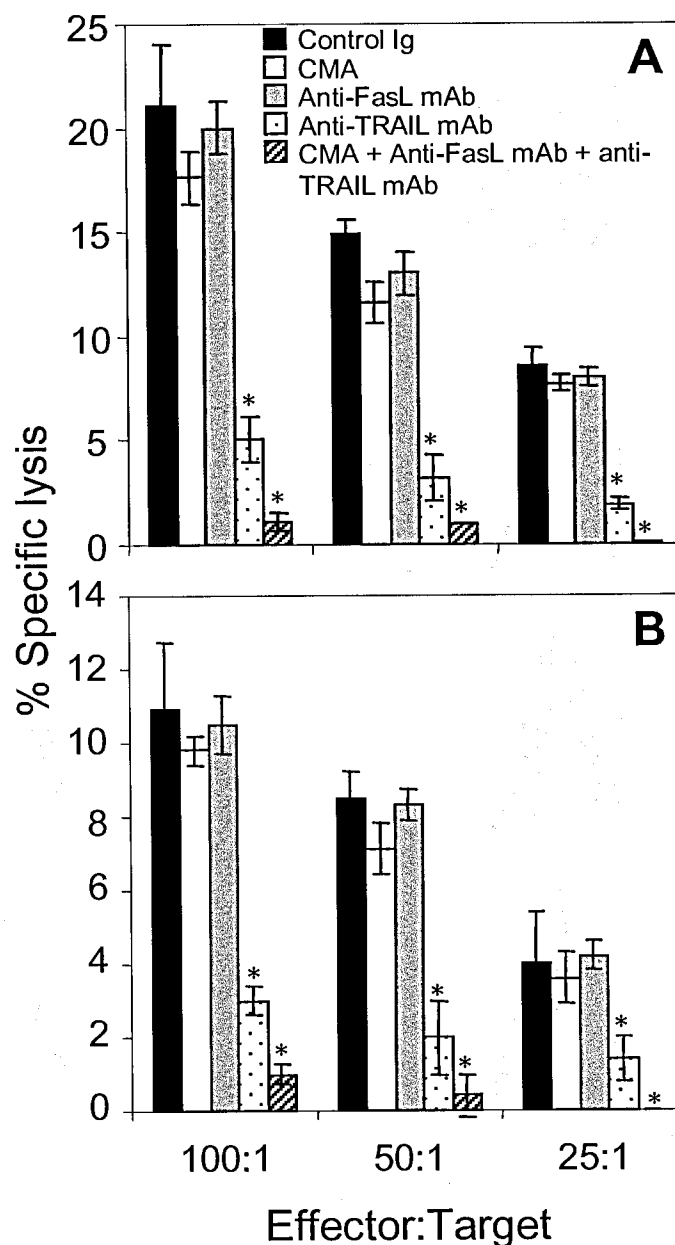


Figure 2.16 Cytolytic activity of liver and spleen MNC from fetal mice. Liver and spleen MNC were isolated from fetal (E17) C57BL/6 RAG-2^{-/-} mice and their cytotoxicity tested against L929 target cells by a 6 h ⁵¹Cr release assay. Assays were set up at three different effector:target ratios, in the presence or absence of 10 µg/ml control Ig, 10 µg/ml anti-mFasL mAb, 10 µg/ml anti-mTRAIL mAb and/or 50 nM CMA. (A) Fetal liver MNC lysis, (B) fetal spleen MNC lysis of L929 cells is shown. Data represent mean ± SEM for triplicate samples. Asterisks indicate groups that are significantly different from control Ig (* P < 0.05). Similar results were obtained in 3 independent experiments.

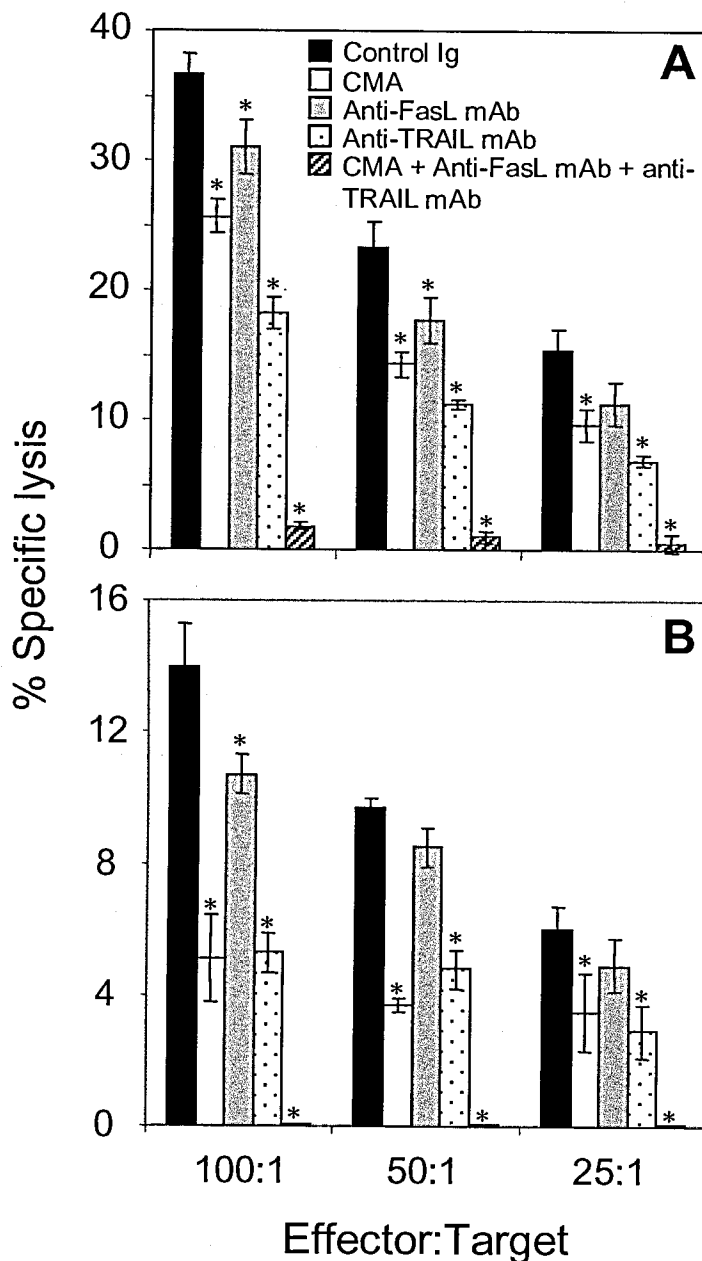


Figure 2.17 Cytolytic activity of liver and spleen MNC from neonatal mice. Liver and spleen MNC were isolated from neonatal C57BL/6 RAG-2^{-/-} mice and their cytotoxicity tested against L929 target cells by a 6 h ⁵¹Cr release assay. Assays were set up at three different effector:target ratios, in the presence or absence of 10 µg/ml control Ig, 10 µg/ml anti-mFasL mAb, 10 µg/ml anti-mTRAIL mAb and/or 50 nM CMA. (A) Neonatal liver MNC lysis, (B) neonatal spleen MNC lysis of L929 cells is shown. Data represent mean ± SEM for triplicate samples. Asterisks indicate groups that are significantly different from control Ig (* P < 0.05). Similar results were obtained in 3 independent experiments.

fetal NK cells, whereas neonatal liver NK cells acquire additional effector mechanisms to induce target cell apoptosis.

Next we examined the cytotoxicity of liver MNC isolated from 2 week old C57BL/6 WT mice against TRAIL-, FasL- and pfp-sensitive DA3 (Figure 2.18A) and L929 (Figure 2.18B) target cells. Liver MNC predominantly exerted TRAIL- and pfp-dependent cytotoxicity that could be blocked by the addition of neutralizing anti-mTRAIL mAb and EGTA, respectively. Weak FasL-dependent cytotoxicity was also observed. This suggested that the effector mechanisms used by neonatal and 2 week old liver NK cells are similar. Liver MNC isolated from 2 week old C57BL/6 mice were also tested against pfp- and FasL-sensitive Yac-1 target cells (Figure 2.18C) and shown to predominantly exert pfp-dependent cytotoxicity that could be blocked by the addition of EGTA, but also weak FasL-dependent cytotoxicity that was blocked by the addition of neutralizing anti-FasL mAb. We additionally analyzed the cytotoxic activity of spleen MNC isolated from 2 week old C57BL/6 mice against TRAIL-, FasL- and pfp-sensitive DA3 target cells (Figure 2.19). Unlike neonatal spleen NK cells that demonstrated TRAIL- and pfp-dependent cytotoxicity, splenocytes isolated from 2 week old mice predominantly exerted pfp-dependent cytotoxicity (Figure 2.19), which is consistent with a decrease in TRAIL expression in the spleen by 2 weeks of age (Figure 2.8). These data suggest that TRAIL-dependent cytotoxicity persists in the liver, but subsides in the spleen by 2 weeks of age.

2.4.9 TRAIL-deficient mice lack constitutive TRAIL expression

Mice genetically deficient in TRAIL were generated by homologous recombination in 129 derived stem cells, as described in Materials and Methods (Section 2.3), and backcrossed n=10 generations to C57BL/6 and BALB/c backgrounds. C57BL/6 TRAIL^{-/-} and BALB/c TRAIL^{-/-} mice displayed no obvious pathological, hematological or reproductive defects (data not shown).

To confirm that C57BL/6 TRAIL^{-/-} mice lacked constitutive TRAIL expression, liver MNC isolated from these mice were stained with antibodies to NK1.1, CD3

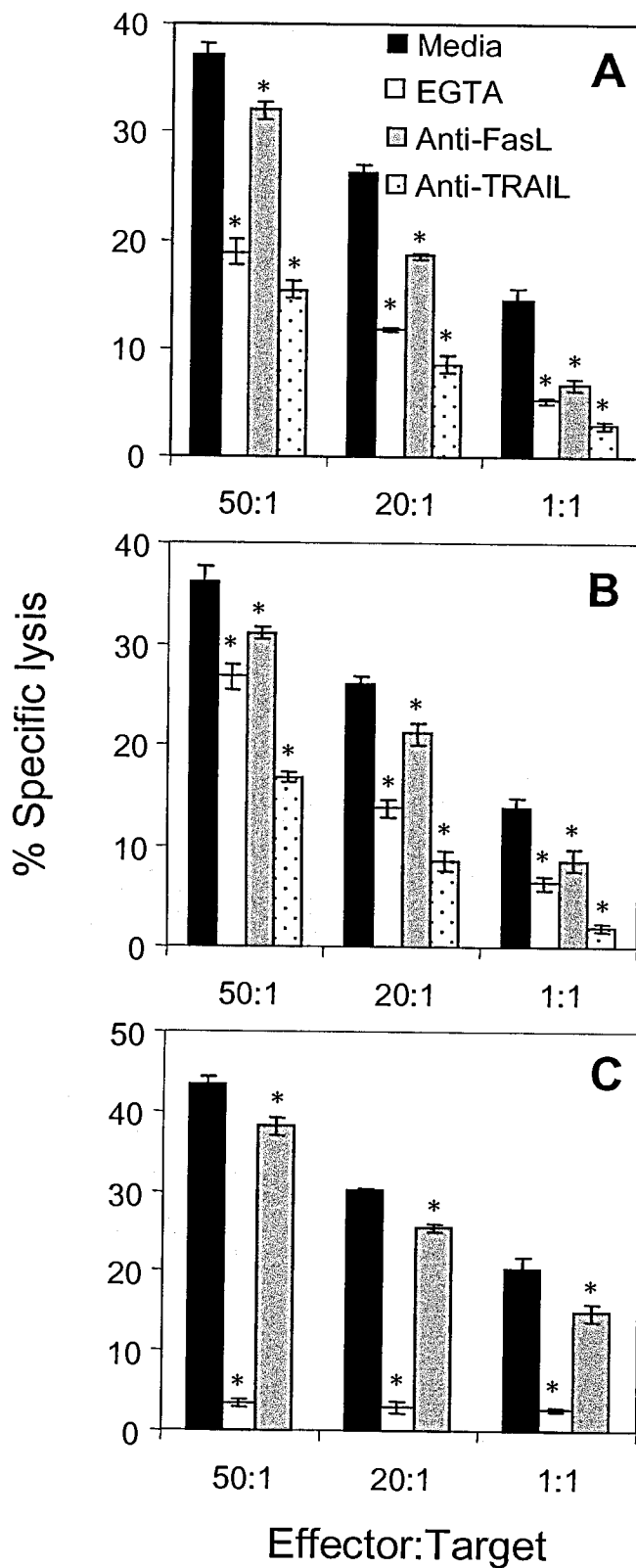


Figure 2.18 Cytolytic activity of liver MNC from 2 week old mice. Liver MNC were isolated from 2 week old C57BL/6 mice and their cytotoxicity tested against (A) DA3, (B) L929 or (C) Yac-1 target cells by a 5 h ^{51}Cr release assay. Assays were set up at three different effector:target ratios, in the presence or absence of 5 mM EGTA, 20 $\mu\text{g}/\text{ml}$ anti-mFasL mAb or 10 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb, or no inhibitor present (media). Data represent mean \pm SEM for triplicate samples. Asterisks indicate groups that are significantly different from media alone (* $P < 0.03$).

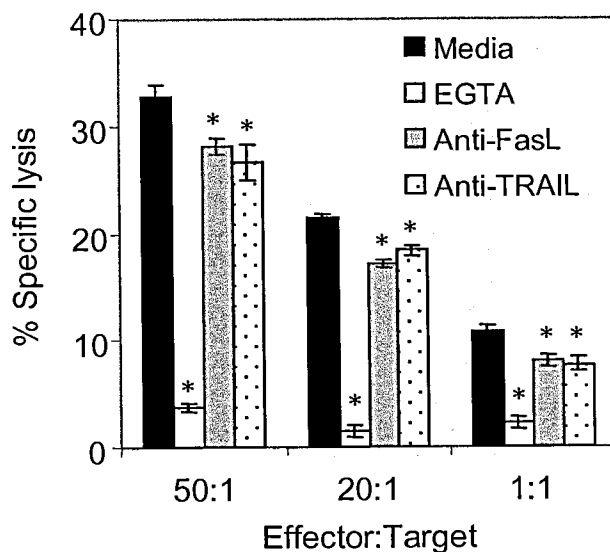


Figure 2.19. Cytolytic activity of spleen MNC isolated from 2 week old mice. Spleen MNC were isolated from 2 week old C57BL/6 mice and their cytotoxicity tested against DA3 target cells by a 5 h ^{51}Cr release assay. Assays were set up at three different effector:target ratios, in the presence or absence of 5 mM EGTA, 20 $\mu\text{g/ml}$ anti-mFasL mAb or 10 $\mu\text{g/ml}$ anti-mTRAIL mAb, or no inhibitor present (media). Data represent mean \pm SEM for triplicate samples. Asterisks indicate groups that are significantly different from media alone (* $P < 0.03$).

and TRAIL or isotype control mAb. C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice displayed similar proportions of liver NK, NKT and T cells (Figure 2.1 and 2.20A). Whilst TRAIL was expressed on a subset of liver NK cells in C57BL/6 mice (Figure 2.1), NK cells isolated from C57BL/6 TRAIL^{-/-} mice completely lacked TRAIL expression, as did NK1.1⁺CD3⁺, NK1.1⁻CD3⁺ and NK1.1⁻CD3⁻ cells (Figure 2.20A). TRAIL expression was also not detected in permeabilized liver MNC from C57BL/6 TRAIL^{-/-} mice (Figure 2.20B). These data confirmed that C57BL/6 TRAIL^{-/-} mice do not express TRAIL protein in the liver.

Although TRAIL expression was not detected in the spleen of adult C57BL/6 WT mice (Figure 2.2), low level TRAIL staining were observed on fetal/neonatal splenic NK cells and in young mice (Figure 2.8). To ensure that C57BL/6 TRAIL^{-/-} completely lacked TRAIL expression, spleen MNC isolated from these mice were analyzed for TRAIL expression. C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice displayed similar proportions of spleen NK, NKT and T cells (data not shown), and TRAIL was not detected on NK1.1⁺CD3⁻, NK1.1⁺CD3⁺, NK1.1⁻CD3⁺ or NK1.1⁻CD3⁻ cells (Figure 2.21A). TRAIL was also not detected in spleen MNC from C57BL/6 TRAIL^{-/-} mice that had been permeabilized (Figure 2.21B). These data confirmed that C57BL/6 TRAIL^{-/-} mice do not express TRAIL protein in the spleen.

To confirm that BALB/c TRAIL^{-/-} mice lacked constitutive TRAIL expression, liver and spleen MNC isolated from these mice were stained with antibodies to IL-2R β , CD3 and TRAIL or isotype control mAb. BALB/c WT and BALB/c TRAIL^{-/-} mice displayed similar proportions of liver NK, NKT and T cells (data not shown). Whilst TRAIL was expressed on a subset of liver NK cells in BALB/c WT mice (Figure 2.6), liver NK cells isolated from BALB/c TRAIL^{-/-} mice lacked TRAIL expression, as did IL-2R β ⁺CD3⁺, IL-2R β ⁻CD3⁺ and IL-2R β ⁻CD3⁻ cells (Figure 2.22A). TRAIL was also not detected on IL-2R β ⁺CD3⁻, IL-2R β ⁺CD3⁺, IL-2R β ⁻CD3⁺ or IL-2R β ⁻CD3⁻ cells in the spleen of these mice (Figure 2.22B). These data confirmed that BALB/c TRAIL^{-/-} mice do not express TRAIL protein in the liver or spleen.

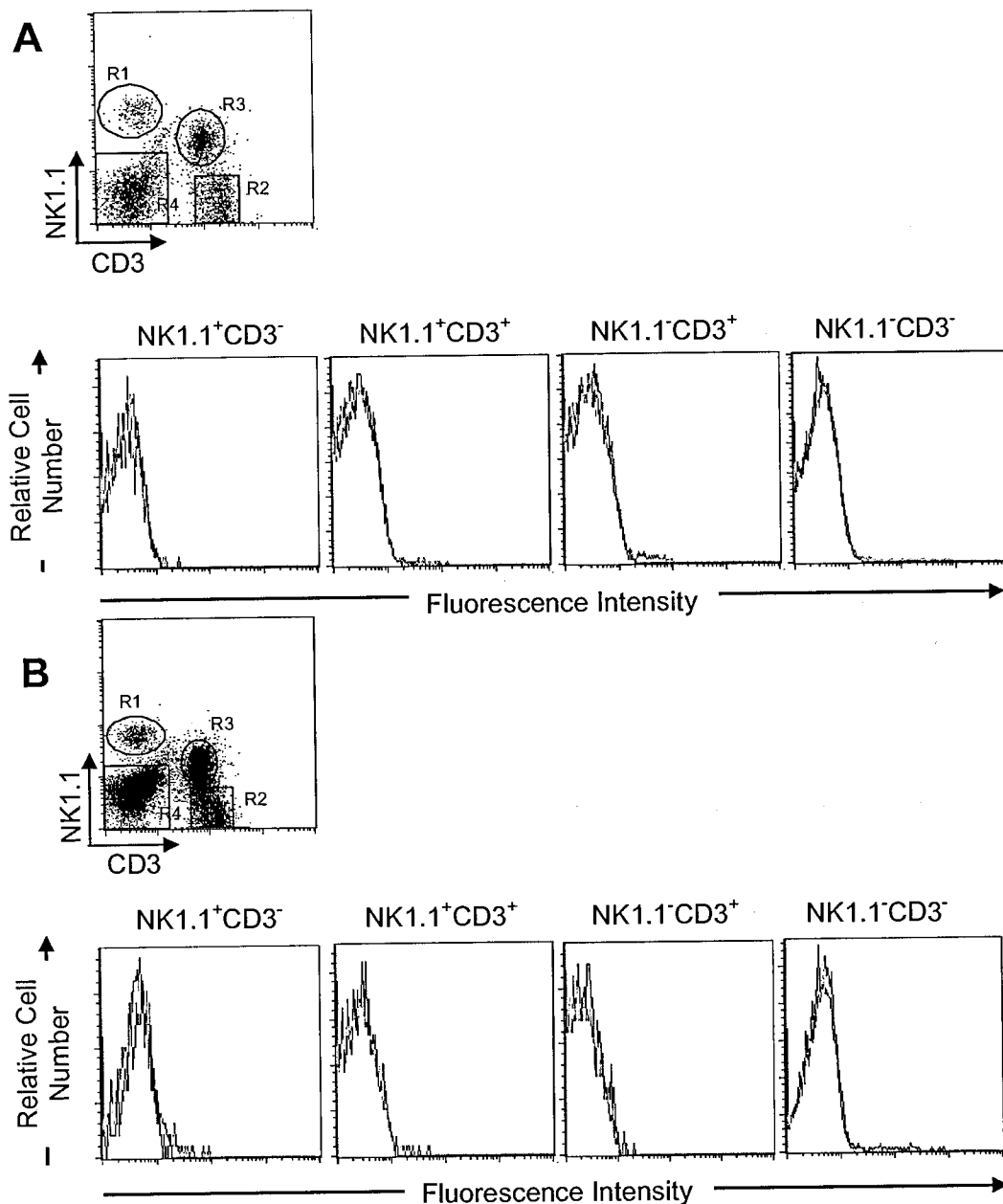


Figure 2.20. Absence of TRAIL expression in the liver of C57BL/6 TRAIL-deficient mice. Liver MNC isolated from adult C57BL/6 TRAIL^{-/-} mice were permeabilized or left unpermeabilized and stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. (A) Representative CD3 versus NK1.1 FACS plot for unpermeabilized liver MNC. The R1 gate comprises NK1.1⁺CD3⁻ cells, R2 comprises NK1.1⁺CD3⁺ cells, R3 comprises NK1.1⁻CD3⁺ cells and R4 comprises NK1.1⁻CD3⁻ cells. Histograms demonstrate TRAIL/isotype control antibody staining on the gated liver MNC populations (R1-R4), for unpermeabilized cells. (B) Representative CD3 versus NK1.1 FACS plot for permeabilized liver MNC. Histograms demonstrate TRAIL/isotype control antibody staining on the gated MNC populations (R1-R4), for permeabilized cells. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Data are representative of the analysis of 5 mice for (A) and 2 mice for (B).

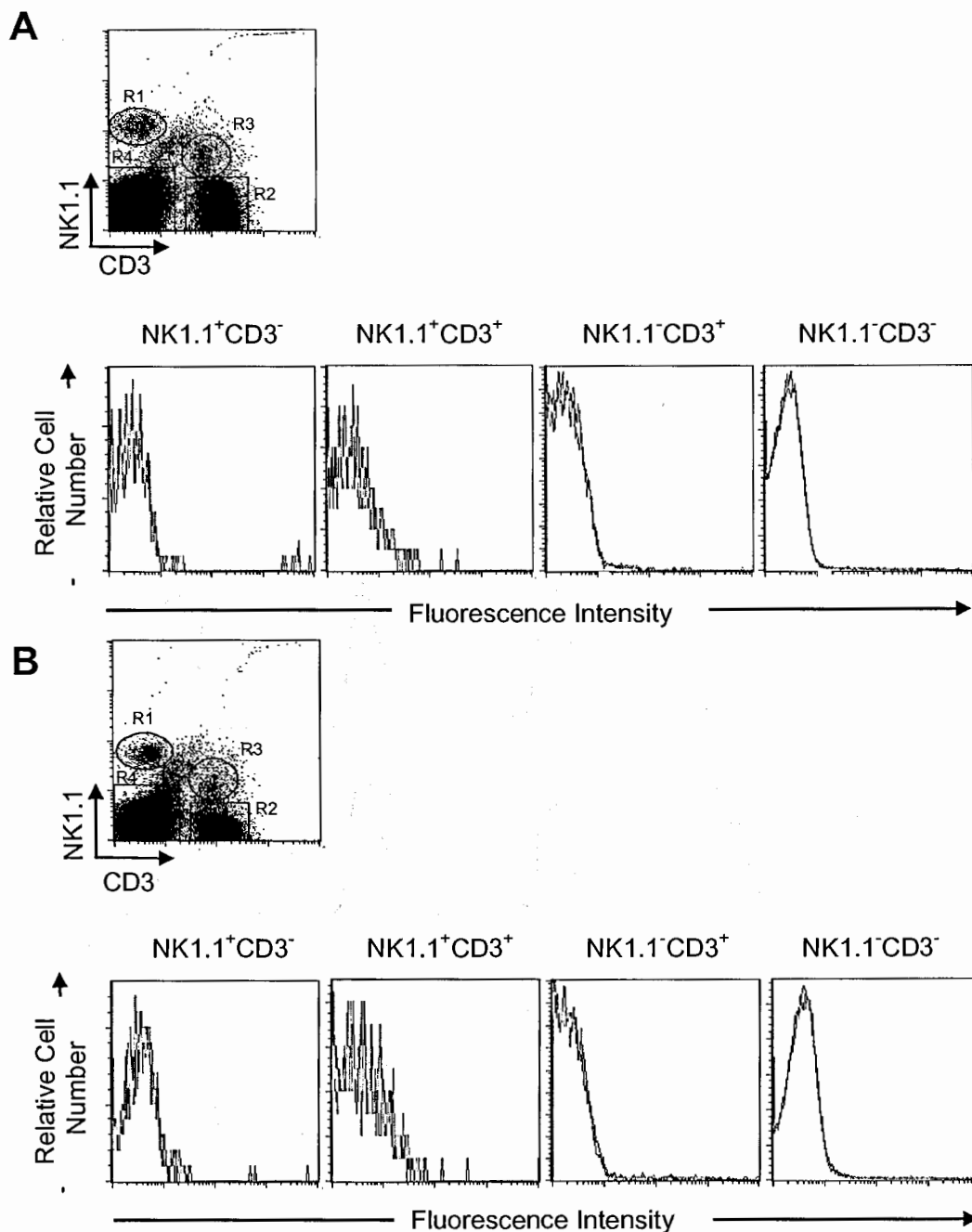


Figure 2.21. Absence of TRAIL expression in the spleen of C57BL/6 TRAIL-deficient mice. Spleen MNC isolated from adult C57BL/6 TRAIL^{-/-} mice were permeabilized or left unpermeabilized and stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. (A) Representative CD3 versus NK1.1 FACS plot for unpermeabilized spleen MNC. The R1 gate comprises NK1.1⁺CD3⁻ cells, R2 comprises NK1.1⁺CD3⁺ cells, R3 comprises NK1.1⁻CD3⁺ cells and R4 comprises NK1.1⁻CD3⁻ cells. Histograms demonstrate TRAIL/isotype control antibody staining on the gated MNC populations (R1-R4), for unpermeabilized cells. (B) Representative CD3 versus NK1.1 FACS plot for permeabilized spleen MNC. Histograms demonstrate TRAIL/isotype control antibody staining on the gated MNC populations (R1-R4), for permeabilized cells. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Data are representative of the analysis of 5 mice for (A) and 2 mice for (B).

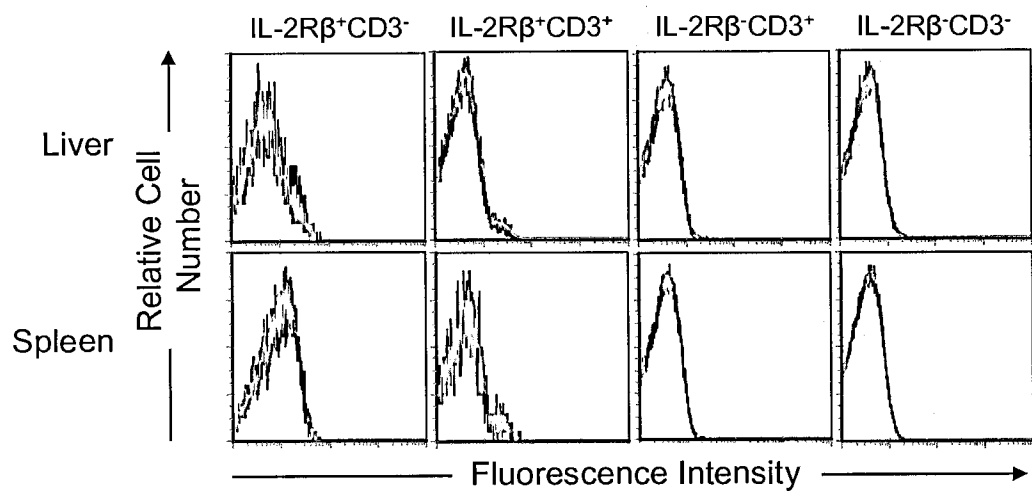


Figure 2.22. Absence of TRAIL expression in the liver and spleen of BALB/c TRAIL-deficient mice. Liver and spleen MNC isolated from adult BALB/c TRAIL^{-/-} mice were stained with antibodies to CD3, IL-2Rβ, DX5 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on gated IL-2Rβ⁺CD3⁻, IL-2Rβ⁺CD3⁺, IL-2Rβ⁻CD3⁺ and IL-2Rβ⁻CD3⁻ liver and spleen MNC. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Data are representative of the analysis of 4 mice.

2.4.10 TRAIL is not essential for NK cell development *in vivo*

As we had shown that TRAIL is typically expressed on immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} NK cells (Figure 2.4), we then assessed whether proportions of immature NK cells were decreased in mice devoid of TRAIL expression. Proportions of immature DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} NK cells were equivalent in C57BL/6 TRAIL^{-/-} compared to C57BL/6 WT mice (Figure 2.23), suggesting that TRAIL is not essential for NK cell development *in vivo*.

2.4.11 TRAIL-mediated cytotoxicity *in vitro*

To confirm that TRAIL-deficient mice lacked TRAIL function, the contribution of TRAIL to the cytotoxicity of hepatic and splenic NK cells was assessed against TRAIL-sensitive 4T1.2 and Renca tumor target cells. The cytotoxicity of liver MNC isolated from BALB/c WT mice, against 4T1.2 (Figure 2.24A) and Renca (Figure 2.25A) tumor targets was partially abrogated by the addition of neutralizing anti-mTRAIL mAb and completely inhibited by the addition of both anti-mTRAIL mAb and the pfp inhibitor CMA. This suggested that lysis of 4T1.2 and Renca tumor targets by WT liver MNC is both TRAIL- and pfp-dependent. The cytotoxicity of liver MNC isolated from BALB/c IFN- γ ^{-/-} and BALB/c TRAIL^{-/-} mice, against 4T1.2 (Figure 2.24A) and Renca (Figure 2.25A) tumor targets was unaffected by the addition of anti-mTRAIL mAb, but completely inhibited by the addition of CMA, suggesting that liver MNC from these mice lacked TRAIL function but retained pfp function. In contrast, the cytotoxicity of liver MNC isolated from BALB/c pfp^{-/-} mice, against 4T1.2 (Figure 2.24A) and Renca (Figure 2.25A) tumor targets was unaffected by the addition of CMA, but completely inhibited by the addition of anti-mTRAIL mAb, suggesting that liver MNC from these mice lacked pfp function, but retained TRAIL function. The cytotoxicity of spleen MNC isolated from BALB/c WT mice against 4T1.2 (Figure 2.24B) and Renca (Figure 2.25B) tumor targets was completely abrogated by CMA, indicating that pfp was the only effector mechanism used by freshly isolated spleen MNC to lyse these tumor targets. Consistent with this data, BALB/c pfp^{-/-} spleen MNC did not lyse 4T1.2 (Figure 2.24B) or Renca (Figure 2.25B) targets.

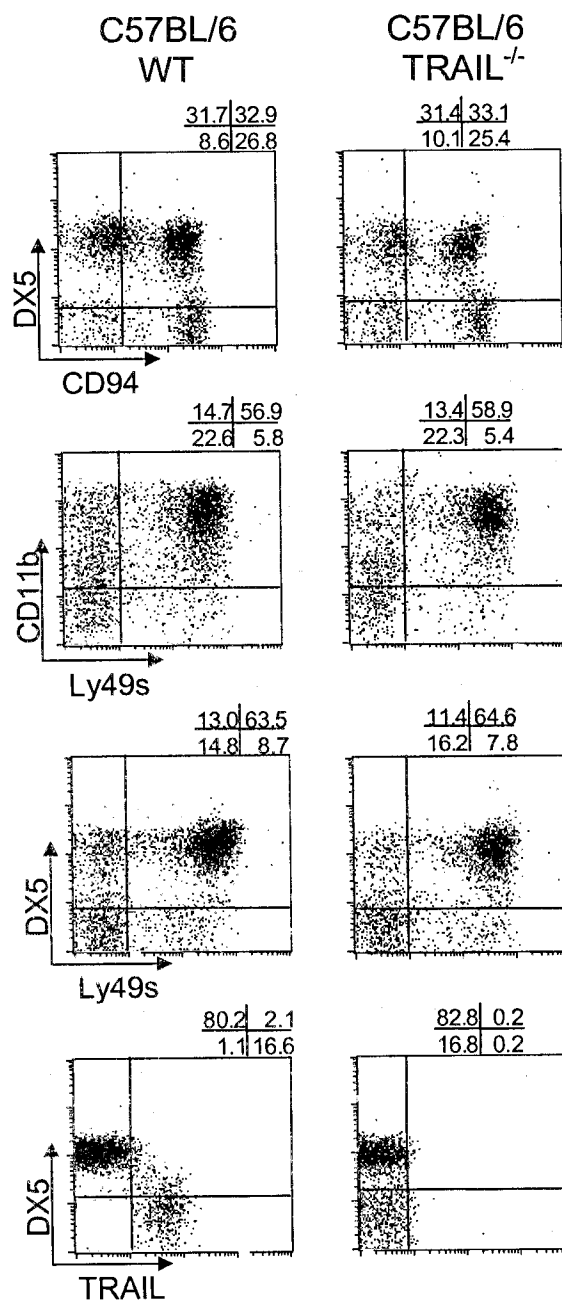


Figure 2.23. NK cell subpopulations in TRAIL-deficient mice. Liver MNC isolated from adult C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice were stained with combinations of antibodies to CD3, NK1.1, DX5, CD94, CD11b, Ly49s (A, C/I, G2, D) and TRAIL. Representative CD94 versus DX5, Ly49s versus CD11b, Ly49s versus DX5 and TRAIL versus DX5 are shown for gated NK1.1⁺CD3⁻ cells. Numbers represent the percentages of cells in each quadrant. Data are representative of 3 independent experiments.

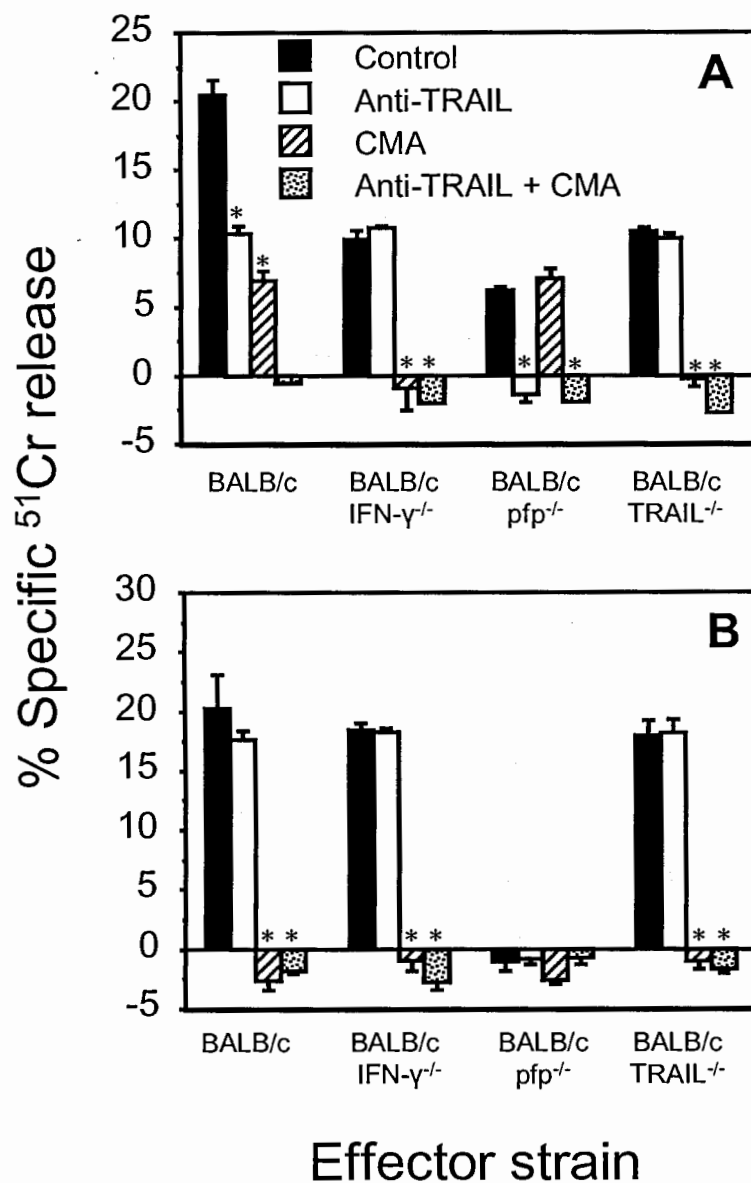


Figure 2.24. TRAIL contributes to NK cell-mediated cytotoxicity of 4T1.2 tumor cells *in vitro*. Liver and spleen MNC were isolated from adult BALB/c, BALB/c IFN- $\gamma^{-/-}$, BALB/c pfp $^{-/-}$ and BALB/c TRAIL $^{-/-}$ mice and their cytotoxic activities tested against 4T1.2 target cells by an 8 h ^{51}Cr release assay. Assays were performed at various effector:target ratios, in the presence or absence of 50 nM CMA, 10 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb, 50 nM CMA plus 10 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb, or no inhibitor present (control). (A) Liver MNC lysis of 4T1.2 target cells is shown at 50:1 effector:target ratio. (B) Spleen MNC lysis of 4T1.2 target cells is shown at 100:1 effector:target ratio. Data are representative of those across the effector:target ratio of 50 to 5:1 (for liver) and 100 to 5:1 (for spleen). Data represent mean \pm SEM of triplicate samples. Asterisks indicate groups that are significantly different to control (* $P < 0.05$). Data are representative of 2 independent experiments.

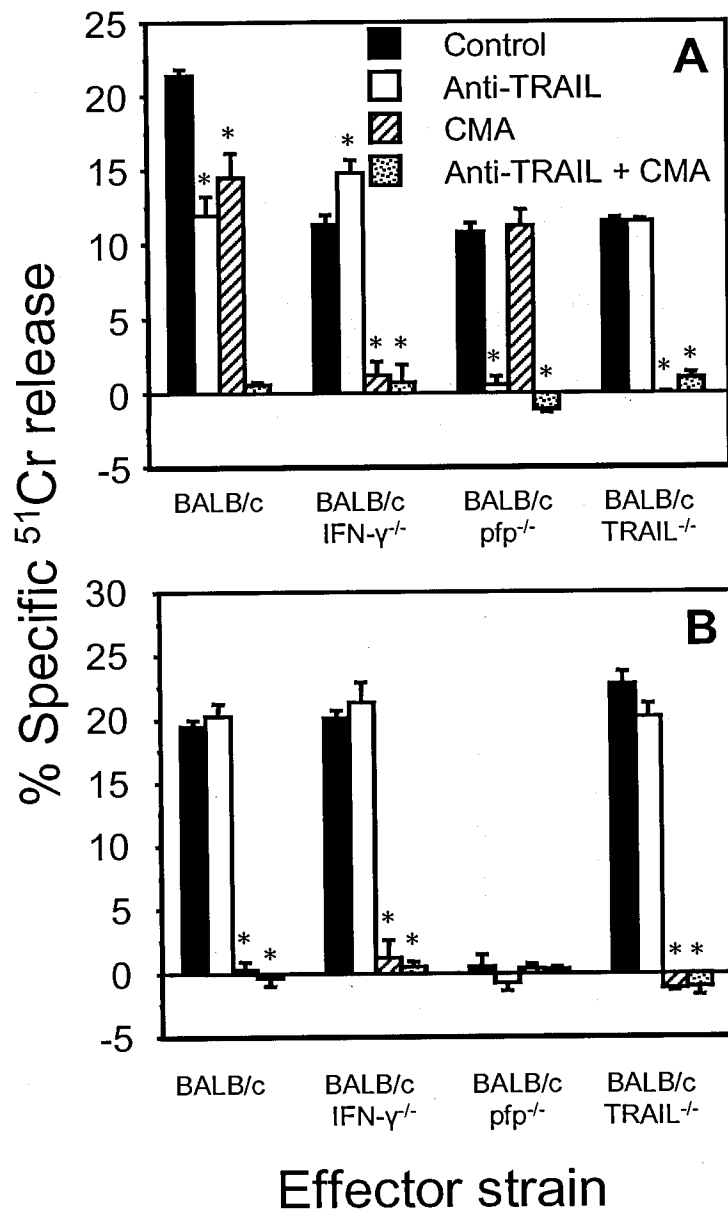


Figure 2.25. TRAIL contributes to NK cell-mediated cytotoxicity of Renca tumor cells *in vitro*. Liver and spleen MNC were isolated from adult BALB/c, BALB/c IFN- $\gamma^{-/-}$, BALB/c pfp $^{-/-}$ and BALB/c TRAIL $^{-/-}$ mice and their cytotoxic activities tested against Renca target cells by an 8 h ^{51}Cr release assay. Assays were performed at various effector:target ratios, in the presence or absence of 50 nM CMA, 10 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb, 50 nM CMA plus 10 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb, or no inhibitor present (control). (A) Liver MNC lysis of Renca targets cells is shown at 50:1 effector:target ratio. (B) Spleen MNC lysis of Renca target cells is shown at 100:1 effector:target ratio. Data are representative of those across the effector:target ratio of 50 to 5:1 (for liver) and 100 to 5:1 (for spleen). Data represent mean \pm SEM of triplicate samples. Asterisks indicate groups that are significantly different to control (* $P < 0.05$). Data are representative of 2 independent experiments.

2.5 Discussion

Our study has significantly extended a previous report that identified constitutive TRAIL expression on a small subset of liver NK cells (Takeda et al., 2001), by demonstrating that TRAIL is expressed on DX5^{lo}CD11b^{lo}Ly49⁻CD94^{hi} liver NK cells, a surface phenotype characteristic of "immature" NK cells (Kim et al., 2002). Further, we identified that TRAIL-expressing NK cells predominate in the liver of fetal, neonatal and young mice, and showed that TRAIL is additionally expressed by fetal and neonatal spleen NK cells and by non-type I liver NKT cells in young mice. Moreover, fetal liver and spleen NK cells predominantly exerted TRAIL-dependent cytotoxicity, suggesting that TRAIL is the first cytotoxic molecule expressed on NK cells. Importantly, we also reported the first characterization of TRAIL-deficient mice generated by gene-targeting, and showed that these mice are devoid of TRAIL expression and function.

A previous report suggested that adult IFN- γ -deficient and IFN- γ -receptor-deficient mice lacked constitutive TRAIL expression on liver NK cells (Takeda et al., 2001), indicating a critical role for IFN- γ in the regulation of TRAIL expression *in vivo*. Herein, we detected impaired TRAIL expression ($5.5 \pm 4.5\%$) on phenotypically immature liver NK cells in adult IFN- γ -deficient mice, compared to adult C57BL/6 WT mice ($18.5 \pm 4.5\%$). Relatively higher proportions of TRAIL-expressing liver NK cells were observed in IFN- γ -deficient mice at 2 and 4 weeks of age ($45.4 \pm 1.4\%$ and $47.3 \pm 2.4\%$ respectively). Therefore, although endogenous host IFN- γ contributes to TRAIL expression on liver NK cells, molecules other than IFN- γ are additionally important, especially in the earlier stages of NK cell development. Our results indicated that IL-12, IL-18, TNF, IL-4, IFN α and IFN β are not solely critical for TRAIL expression *in vivo*. Using germ-free mice, our collaborators have recently shown that TRAIL-expression by liver NK cells is normal and not dependent on endogenous bacterial flora (personal communication Dr. Kazuyoshi Takeda).

TRAIL is expressed on liver NK cells isolated from scid and RAG-2-deficient mice, suggesting that the IFN- γ required for constitutive TRAIL expression in the liver is not produced by conventional T cells or NKT cells (Takeda et al., 2001). The majority of TRAIL-expressing liver NK cells produce IFN- γ , suggesting that TRAIL expression on liver NK cells may be regulated by IFN- γ secreted from NK cells in an autocrine manner (Takeda et al., 2001). However, this does not explain TRAIL expression on the small proportion of liver NK cells that do not produce IFN- γ , and suggests that an additional source of IFN- γ is required. Although IL-12 can stimulate NK cells and macrophages to secrete IFN- γ (Kodama et al., 1999; Puddu et al., 1997; Takeda et al., 2000), TRAIL expression in IL-12-deficient mice was equivalent to WT mice, suggesting that IL-12 does not regulate TRAIL expression *in vivo*. The alternate source of IFN- γ required for constitutive TRAIL expression *in vivo* is yet to be determined.

In the present study, we showed that constitutive TRAIL expression on IL-2R β^+ CD3 $^-$ liver NK cells was higher in C57BL/6 WT compared to BALB/c WT mice. In Chapter 3, we tested a panel of mouse tumor cell lines for sensitivity to TRAIL-mediated killing. Whilst a large proportion of BALB/c-derived tumor cell lines were TRAIL-sensitive, C57BL/6-derived tumor cell lines were largely resistant to TRAIL-dependent killing. On the basis of immunoediting (Dunn GP et al., 2002; Dunn GP et al., 2004), one might postulate from these findings that decreased constitutive TRAIL expression in BALB/c WT mice predisposes them to development of TRAIL-sensitive tumors that are typically eliminated in C57BL/6 WT mice. Consistent with this hypothesis, TRAIL-sensitive tumors preferentially emerged in anti-TRAIL mAb-treated p53 $^{+/-}$ mice and in anti-mTRAIL mAb-treated WT mice inoculated with MCA to develop fibrosarcomas (Takeda et al., 2002). Moreover, recent studies in our laboratory have shown that approximately 20% of C57BL/6 TRAIL $^{-/-}$ mice develop lymphoma and leukemia with age, suggesting that TRAIL-deficiency predisposes mice to tumor development *in vivo* (personal communication, Dr. Mark J Smyth). It might now be of interest to examine whether TRAIL expression on liver NK cells varies significantly between a number of mouse strains.

A linear differentiation model for human NK cell development has been proposed by Perussia and colleagues (Loza and Perussia, 2001) (Figure 1.1). Briefly, 'Immature' (type 2) IL-13/IL-5-producing, TRAIL-expressing NK cells differentiate into 'Intermediate' (type 0) NK cells when cultured in the presence of IL-12. These 'Intermediate' (type 0) NK cells can produce both IL-13 and IFN- γ and exert TRAIL-, FasL- and pfp-mediated cytotoxicity. IL-12 stimulated 'Intermediate' (type 0) NK cells further differentiate into 'Mature' (type 1) NK cells that produce IFN- γ and mediate apoptosis in a FasL- and pfp-dependent manner. Therefore, TRAIL⁺ mouse fetal liver and spleen NK cells that predominantly exerted TRAIL-dependent cytotoxicity, and do not secrete IFN- γ or IL-13 (personal communication, Dr. Kazuyoshi Takeda), might correspond to 'Immature' (type 2) human NK cells. TRAIL⁺ liver NK cells in adult mice that exerted TRAIL-, FasL- and pfp-mediated cytotoxicity might correspond to 'Intermediate' (type 0) human NK cells. Importantly, phenotypic analysis of NK cells isolated from TRAIL-deficient mice revealed normal proportions of immature and mature NK cells, suggesting that although TRAIL is expressed on phenotypically immature NK cells, TRAIL expression is not required for NK cell development.

Cytotoxic molecules expressed on immature NK cells that lack the majority of Ly49 inhibitory receptors might be expected to cause non-specific apoptosis and tissue damage in young mice. CD94/NKG2 recognizes the non-classical MHC class I molecule Qa-1^b (Sivakumar et al., 1999; Vance et al., 1999; Vance et al., 1998), is expressed at high levels by fetal splenic NK cells (Sivakumar et al., 1999; Van Beneden et al., 2001), and has been suggested to play a role in the maintenance of self-tolerance during fetal life (Salcedo et al., 2000; Sivakumar et al., 1999; Van Beneden et al., 2001). Nonetheless, the selective ability of TRAIL to kill transformed and virus-infected cells, but not most normal self-cells, might render TRAIL a comparatively "safe" effector molecule to express on immature NK cells, compared to other less selective cytotoxic molecules such as FasL and pfp. Although it is unknown why constitutive TRAIL expression is elevated in young mice and downregulated after birth, TRAIL may be required as a

supporting effector molecule during the period that the adaptive immune system is being matured in young mice. This would be important for host resistance to virus and pathogens encountered early in life, as dependence upon maternal immunity diminishes. Moreover, the challenges encountered by the neonate might differ from those encountered in the adult, and might require different mechanisms of immune defense. Alternatively, while IFNs can stimulate TRAIL expression on a number of cell types in adult mice, young mice are exquisitely sensitive to IFN- γ toxicity (Gresser et al., 1981), and may require a higher level of constitutive TRAIL expression on NK cells to avoid toxicity by IFN- γ -mediated stimulation.

Whilst we have identified constitutive TRAIL expression on fetal and neonatal spleen NK cells, and on non-type I liver NKT cells in young mice, TRAIL expression is not retained on these cells later in life. Adoptive transfer of TRAIL⁺ NK cells isolated from adult or neonatal liver, or neonatal spleen, resulted in the appearance of TRAIL⁺ NK cells in the liver or spleen of recipient mice (personal communication, Dr. Kazuyoshi Takeda). This suggested that immature TRAIL⁺ NK cells are able to further differentiate into mature TRAIL⁺ NK cells. The reason TRAIL expression is uniquely retained on a subpopulation of liver NK cells remains to be determined. The micro-environment of the liver, as opposed to the spleen, might facilitate the maintenance of TRAIL⁺ NK cells, since signals from stromal cells are known to support NK cell differentiation *in vivo* (Colucci et al., 2003; Iizuka et al., 1999; Roth et al., 2000; Williams et al., 1999). Alternatively, the unique pattern of cytokine and chemokine receptor, and/or adhesion molecule expression on TRAIL⁺ NK cells might enable their retention in the liver, since cytokines and chemokines critically contribute to NK cell development (Colucci et al., 2003).

The TRAIL-deficient mice generated in the present study are a unique tool that will be exploited in the remaining chapters of this thesis to determine the role of TRAIL in regulation of tumor development, homeostasis of the immune system, and control of autoimmune disease induction.

CHAPTER THREE

THE ROLE OF TRAIL IN NK CELL-MEDIATED TUMOR IMMUNITY

3.1 Abstract

Although rTRAIL can induce significant tumor regression *in vivo* without systemic toxicity, less is known about the role natural TRAIL plays in tumor surveillance *in vivo*. Using a 2PK3-mTRAIL transfectant previously shown to induce TRAIL-dependent cytotoxicity, we examined the susceptibility of 16 murine tumor cell lines to TRAIL-mediated apoptosis *in vitro*. We identified the BALB/c-derived mammary carcinomas 4T1.2, DA3 and EMT6.5 to be particularly TRAIL-sensitive. Using the TRAIL gene-targeted mice characterized in Chapter 2, we now demonstrate that TRAIL plays an important role in the suppression of 4T1.2 and DA3 tumor initiation and metastasis, and the therapeutic efficacy of α -GalCer. Previous studies in our laboratory have shown that NKG2D-mediated tumor rejection is pfp-dependent. Using DA3-H60 retroviral infectants, we now demonstrate that TRAIL plays a minor role in NKG2D-mediated tumor rejection in the lung. This study is amongst the first to indicate an important and general role for TRAIL in the host defense against transformed cells. Our data support the notion that TRAIL, or stimulating the TRAIL pathway, may prove effective as a cancer therapy *in vivo*.

3.2 Introduction

The anti-tumor activity of rTRAIL has been clearly demonstrated *in vivo* against human colon carcinoma (Ashkenazi et al., 1999; LeBlanc et al., 2002), glioma (Fulda et al., 2002; Pollack et al., 2001), multiple myeloma (Mitsiades et al., 2001) and breast carcinoma xenografts (Walczak et al., 1999). rTRAIL has also been reported to induce apoptosis in established xenografts (Ashkenazi et al., 1999; Walczak et al., 1999), and to suppress the growth of xenografts of human tumor cells isolated from patients and grown only briefly in culture before transplantation into mice (Ashkenazi, 2002). Moreover, rTRAIL acts synergistically with ionizing radiation (Chinnaiyan et al., 2000) and certain chemotherapeutic drugs (Ashkenazi et al., 1999; Nagane et al., 2000) to cause regression and even remission of established tumor xenografts *in vivo*. Together these studies reveal the potential of rTRAIL as a cancer therapeutic, and highlight the importance of in depth studies to investigate the role of endogenous TRAIL in tumor surveillance.

NK cells can mediate spontaneous killing of MHC class I-deficient tumor cells (Ljunggren and Karre, 1985; van den Broek et al., 1995), allowing them to kill cancerous host cells that have downregulated class I MHC expression in order to evade the adaptive immune response (Algarra et al., 2000). NK cells have also been established to play a role in control of tumor metastasis *in vivo* (Karre et al., 1986; Smyth et al., 1999; Talmadge et al., 1980). TRAIL is constitutively expressed by a small proportion of liver NK cells in adult mice (Takeda et al., 2001), and TRAIL expression can be induced on IL-2-, IL-15- and IFN-stimulated NK cells (Kayagaki et al., 1999; Zamai et al., 1998). Recent studies in our laboratory have demonstrated that TRAIL blockade, using a neutralizing anti-mTRAIL mAb, increased L929, LB27.4 and Renca experimental liver metastases *in vivo* (Takeda et al., 2001). Moreover, the anti-metastatic effect of TRAIL was not observed in mice depleted of NK cells, or in IFN- γ ^{-/-} mice that express low levels of TRAIL, suggesting that TRAIL contributes to NK cell-mediated tumor surveillance in the liver (Takeda et al., 2001).

NK cell function is controlled by a balance of NK cell inhibitory and activating signals (Biassoni et al., 2001; Campbell and Colonna, 2001; Diefenbach and Raulet, 1999; Diefenbach and Raulet, 2001; Lanier, 2000; Lanier, 2001). NK cell inhibitory receptors bind to self-MHC class I molecules, whereas NK cell activating receptors bind to ligands expressed on stressed, transformed, and virus-infected cells (Biassoni et al., 2003). The NKG2D receptor is a type II disulphide-linked dimer that contains a lectin-like extracellular domain (Ding et al., 1999). In the mouse, NKG2D is expressed on freshly isolated NK cells, but also on LAK cells, activated CD8⁺ T cells and macrophages as well as subsets of NKT and $\gamma\delta$ T cells (Jamieson et al., 2002; Diefenbach et al., 2000). NKG2D recognizes antigens expressed on stressed, virus- and bacteria-infected, abnormal or cancerous cells (Groh et al., 1998; Vetter et al., 2002), and provides both stimulatory and co-stimulatory signals to NK cells (Diefenbach et al., 2000; Diefenbach et al., 2002; Gilfillan et al., 2002). Murine NKG2D recognizes the minor histocompatibility molecule H60, members of the Rae-1 family (Cerwenka et al., 2000; Diefenbach et al., 2001) and the ULBP-like molecule, MULT-1 (Carayannopoulos et al., 2002). Rejection of NKG2D ligand expressing tumor cells *in vivo* is mediated by NK and CD8⁺ T cells (Cerwenka et al., 2001; Diefenbach et al., 2001), and is pfp-dependent (Hayakawa et al., 2002). NKG2D-mediated rejection of H60 expressing tumors is yet to be examined *in vivo*, and the contribution of the TNF-superfamily to NKG2D-stimulated tumor rejection is unknown.

Herein, we use the 4T1.2 and DA3 mouse models of breast cancer metastasis, and the TRAIL gene-targeted mice characterized in Chapter 2, to substantiate a role for TRAIL in anti-tumor immunity *in vivo*. We additionally demonstrate that TRAIL contributes to the therapeutic efficacy of α -GalCer in the mammary gland and liver, and show that TRAIL plays a minor role in NKG2D-mediated tumor rejection of lung metastasis *in vivo*. Together, our findings indicate that TRAIL plays an important and general role in tumor surveillance *in vivo*.

3.3 Materials and Methods

Cell culture and reagents

The BALB/c-derived renal adenocarcinoma cell line, Renca, the BALB/c-derived mammary carcinoma cell line, DA3, and 6-TG-resistant BALB/c-derived mammary carcinoma cell line, 4T1.2 were grown and maintained as detailed in Chapter 2, Section 2.1, Materials and Methods. The C57BL/6-derived T lymphoma cell line, EL4, the C57BL/6-derived prostate carcinoma cell line, RM-1, the Lewis lung carcinoma cell line, 3LL (kindly provided by Dr. Robert Wilttrout, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD, USA), the BALB/c IFN- γ ^{-/-} MCA-induced tumors, IFN- γ -MCA2 and IFN- γ -MCA3, the BALB/c MCA-induced tumors, BCMCA3 and BCMCA4, and the C57BL/6 pfp^{-/-} p53^{+/-} PN53H1 tumor cell line (kindly provided by Shayna Street, Peter Mac) were maintained at 37°C, 10% CO₂ in DMEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine. The BALB/c-derived mammary carcinoma cell line DA3 retrovirally infected with H60 (DA3-H60) or vector control (DA3-MSCV) (kindly provided by Janice Kelly, Peter Mac), the class I-deficient C57BL/6-derived lymphoma cell line, RMA-S, the B cell lymphoma cell line A20 (kindly provided by Dr. Thomas J. Sayers, Frederick, MD, USA), the p53^{-/-} T cell lymphoma cell lines KO52-F11 and KO52-DA20 and the murine B lymphoma cell line 2PK3 and 2PK3 transfected with mouse TRAIL (2PK3-mTRAIL) (kindly provided by Dr. Hideo Yagita, Juntendo University School of Medicine, Tokyo, Japan) were maintained at 37°C, 10% CO₂ in RPMI 1640 supplemented with 10% (v/v) FCS and 2 mM L-glutamine. The BALB/c derived breast carcinoma cell line EMT6.5 (kindly provided by Nicholas Clements, Peter Mac) was maintained at 37°C, 10% CO₂ in α -MEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine. α -GalCer and control vehicle, and the anti-mTRAIL mAb (N2B2) were obtained and prepared as detailed in Chapter 2, Section 2.1, Materials and Methods.

Mice

Inbred BALB/c WT mice were purchased from The WEHI. BALB/c TRAIL^{-/-}, BALB/c pfp^{-/-} and BALB/c IFN- γ ^{-/-} mice were obtained and bred at Peter Mac as

detailed in Chapter 2, Section 2.1, Materials and Methods. BALB/c pfp and TRAIL-deficient (BALB/c pfp^{-/-} TRAIL^{-/-}) mice were established at Peter Mac by crossing BALB/c pfp^{-/-} and BALB/c TRAIL^{-/-} mice and subsequently interbreeding the F₁ generation. Adult mice 7-12 weeks of age were used in all experiments, under specific pathogen-free conditions according to Animal Experimental Ethics Committee guidelines and approval.

Flow cytometric analysis

The following mAb were used in multi-parameter flow cytometric analysis: anti-mTRAIL-PE, rat IgG2a isotype control-PE, anti-H-2D^d-biotin (34-5-8S) and anti-DR5-biotin (MD5-1) followed by incubation with streptavidin-PE. To avoid non-specific binding of mAbs to FcγR, anti-mouse CD16/32 (2.4G2) mAb was added to the mAb cocktail. Anti-mTRAIL-PE (N2B2) was obtained from e-Bioscience, anti-DR5-biotin was kindly provided by Dr. Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan) and the remaining reagents were sourced from BD Biosciences Pharmingen. NKG2D tetramer and T22 control tetramer were kindly provided by Dr. David Raulet (University of California, Berkeley, CA, USA). After washing the cells with PBS/FCS/azide, cells were analyzed on a FACScan[™] or FACSDiva[™] and the data processed by either CELLQuest[™] or FCS-Express 2 software.

MNC preparation

Mice were treated i.p. with 2 μg α-GalCer on days 0 and 4, prior to isolation of spleen and hepatic MNC on day 5. Liver and spleen MNC were isolated and prepared as detailed in Chapter 2, Section 2.1, Materials and Methods.

Cellular cytotoxicity assay

The cytotoxic activities of the 2PK3 parental and 2PK3-mTRAIL transfectants were determined by ⁵¹Cr-release assay as detailed in Chapter 2, Section 2.1, Materials and Methods. ⁵¹Cr-labelled targets were placed in 96-well plates (2 x 10⁴ cells/well) in 200 μl total volume and incubated in the presence of 2PK3 parental or 2PK3-mTRAIL transfectants at various effector to target cell ratios for 18 h (37°C, 5% CO₂). The assay was also performed in the presence of

purified anti-mTRAIL (N2B2) mAb (1 μ g/ml). Percent specific TRAIL-mediated lysis was determined by subtracting % specific lysis by 2PK3 parental cells from % specific lysis by 2PK3-mTRAIL transfected cells.

The cytotoxic activity of spleen and liver MNC against Renca and 4T1.2 tumor targets was tested in an 8 h 51 Cr-release assay as detailed in Chapter 2, Section 2.1, Materials and Methods. The assay was performed in the presence of purified control rat IgG2a (R35-95; 10 μ g/ml), anti-mTRAIL mAb (N2B2; 10 μ g/ml), and/or CMA (50 nM).

4T1.2 mammary carcinoma growth and metastasis

To examine 4T1.2 tumor growth within the peritoneum, female BALB/c WT, BALB/c TRAIL^{-/-}, BALB/c pfp^{-/-}, BALB/c IFN- γ ^{-/-} and BALB/c pfp^{-/-} TRAIL^{-/-} mice were inoculated i.p. with between 1 to 10,000 4T1.2 cells and monitored for ascites development. Some mice were additionally injected with anti-mTRAIL mAb (N2B2; 500 μ g i.p.) 3 times weekly. To examine primary tumor growth and spontaneous metastasis, female BALB/c WT or BALB/c TRAIL^{-/-} mice were inoculated s.c. in the abdominal mammary gland with 4T1.2 tumor cells at the doses indicated on day 0. Some groups of mice received either: anti-mTRAIL mAb (N2B2; 250 μ g i.p.) on days 0, 1, 4, 7, 10, 14 and 21; anti-asialoGM1 antibody (Wako Pure Chemicals, 200 μ g i.p.) on days -1, 0, 7, and 14; and/or α -GalCer (2 μ g i.p.) on days 0, 4, 8, 12 and 16. Primary tumors were measured every 4 days following tumor inoculation over the course of 30 days with a caliper square as the product of two perpendicular diameters (cm²). Mice were sacrificed at 30 days and spontaneous metastasis in the liver and lung measured by colony assay. Liver samples were finely minced and digested in 5 ml enzyme cocktail (1 x PBS, 0.01% BSA, 1 mg/ml hyaluronidase and 1 mg/ml collagenase type 1, 20 min., 37°C). Lung samples were finely minced and digested in 5 ml enzyme cocktail (1 x PBS, 1 mg/ml collagenase type 4, 6 units/ml elastase, 1 h, 4°C). Digested samples were filtered through a 70 μ m nylon mesh and washed 3 times with 1 x HBSS. Cells were then plated out neat or serially diluted in tissue culture dishes, in medium containing 60 μ M 6-TG for clonogenic growth. 6-TG-resistant tumor cells formed foci within 10-14 days and were fixed with methanol

and stained with 0.03% methylene blue for counting. Clonogenic metastases were calculated on a per organ basis.

DA3 mammary carcinoma growth and metastasis

To examine DA3 parental primary tumor growth, female BALB/c WT and BALB/c TRAIL^{-/-} mice were injected s.c. in the hind flank with 5×10^5 to 3×10^6 DA3 cells and the tumors measured every other day with a caliper square for a period of 36 days. To examine DA3-MSCV and DA3-H60 primary tumor growth, female BALB/c WT mice were injected s.c. with 5×10^5 DA3 or DA3-H60 cells and the tumors measured every other day with a caliper square for a period of 45 days. At the termination of the experiment, mice were sacrificed and spontaneous metastasis measured by harvesting the lungs, fixing them in Bouin's solution (75 ml saturated picric acid, 25 ml 40% formaldehyde, 5 ml glacial acetic acid) overnight and counting the number of metastases with the aid of a dissecting microscope. BALB/c WT, BALB/c TRAIL^{-/-}, BALB/c pfp^{-/-} and BALB/c pfp^{-/-} TRAIL^{-/-} mice were also injected i.v. with between 5×10^4 to 5×10^5 DA3 or DA3-H60 cells, sacrificed after 14 days and the number of lung metastases quantified.

RNA isolation and RT PCR

To isolate total cellular RNA, 1×10^7 DA3 or Renca cells were lysed in 1 ml TRIzol (Invitrogen Life Technologies, Gaithersburg, MD, USA) and incubated at RT for 5 min. A further 1 ml TRIzol and 0.2 ml CHCl₃ were added, the cells shaken vigorously for 15 seconds and incubated at RT for 3 min. After centrifugation (12,000 x g, 15 min., 4°C), the aqueous phase was transferred to a clean tube and the RNA precipitated with 1 ml isopropyl alcohol (10 min., RT). The tube was then centrifuged (12,000 x g, 15 min., 4°C), the supernatant removed and the RNA washed with 1 ml 70% ethanol. The RNA pellet was then air-dried for 10 min., and resuspended in 100 µl RNase-free water (DEPC-treated). Reverse transcription was performed using 2 µg of total RNA in a first-strand cDNA synthesis reaction with Murine Reverse Transcriptase (New England BioLabs Inc, Beverly, MA, USA). PCR was then performed using 7 µl of the RT product. The following oligonucleotides specific for H60 sequences

were used in the PCR: 5' primer, 5'-CTCATGGCAAAGGGAGCCAC C-3'; and 3' primer, 5'-CTATTTTTTTTCTTCAGCATACACCAAGCG-3'. The PCR (30 cycles) was conducted in a Peltier thermal cycler PTC-200 (MJ Research Inc, Watertown, MA, USA). Each cycle included denaturation (95°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 2 min). The initial denaturation period was 5 min. and the final extension was 7 min. As a negative control, the RT product was omitted from the PCR reaction. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, and visualized by ethidium bromide staining under UV illumination in an ImageMaster VDS (Pharmacia Biotech).

Statistical Analysis

Data were analyzed using a two tailed, non-parametric, unpaired Mann Whitney test. P values < 0.05 were considered significant.

3.4 Results

3.4.1 Sensitivity of mouse tumor cell lines to TRAIL-mediated cytotoxicity

Although a number of TRAIL-sensitive human tumor cells lines have been identified (Pitti et al., 1996; Wiley et al., 1995), less is known about the sensitivity of mouse tumor cell lines to TRAIL-mediated cytotoxicity. We screened a panel of mouse tumor cell lines for their sensitivity to TRAIL-mediated cytotoxicity *in vitro*, using a 2PK3-mTRAIL transfectant previously demonstrated to induce TRAIL-dependent apoptosis (Kayagaki et al., 1999). Initially, cell surface expression of mTRAIL was verified on the 2PK3-mTRAIL transfectant cells and shown to be absent on 2PK3 parental cells (Figure 3.1A). To confirm that 2PK3-mTRAIL cells induced TRAIL-dependent cytotoxicity *in vitro*, transfectant cells were tested for their ability to lyse TRAIL-sensitive Renca tumor target cells in an 18 h ^{51}Cr release assay. As expected, 2PK3-mTRAIL cells significantly lysed Renca targets, even at low effector to target ratios (Figure 3.1B), and addition of neutralizing anti-mTRAIL mAb completely abrogated cytotoxicity (Figure 3.1C). In contrast, no significant cytotoxicity was observed with 2PK3 parental cells (Figure 3.1B). The sensitivity of a number of mouse tumor cell lines to TRAIL-mediated cytotoxicity was then compared relative to Renca at an effector to target ratio of 50:1. The BALB/c-derived mouse mammary carcinomas 4T1.2, DA3 and EMT6.5 were found to be particularly TRAIL-sensitive, whilst a number of tumor cell lines including the C57BL/6-derived RMA-S lymphoma, and Lewis lung carcinoma 3LL were TRAIL-resistant (Figure 3.2) (Appendix II). Interestingly, the majority of BALB/c-derived tumor cell lines were TRAIL-sensitive, whilst the majority of C57BL/6-derived tumor cell lines were relatively TRAIL-resistant. To verify this trend, greater numbers of BALB/c- and C57BL/6-derived mouse tumor cell lines need to be tested for their sensitivity to TRAIL-mediated killing.

3.4.2 TRAIL-mediated suppression of 4T1.2 tumor growth

The 4T1.2 mouse mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice (Aslakson and Miller, 1992; Miller et al.,

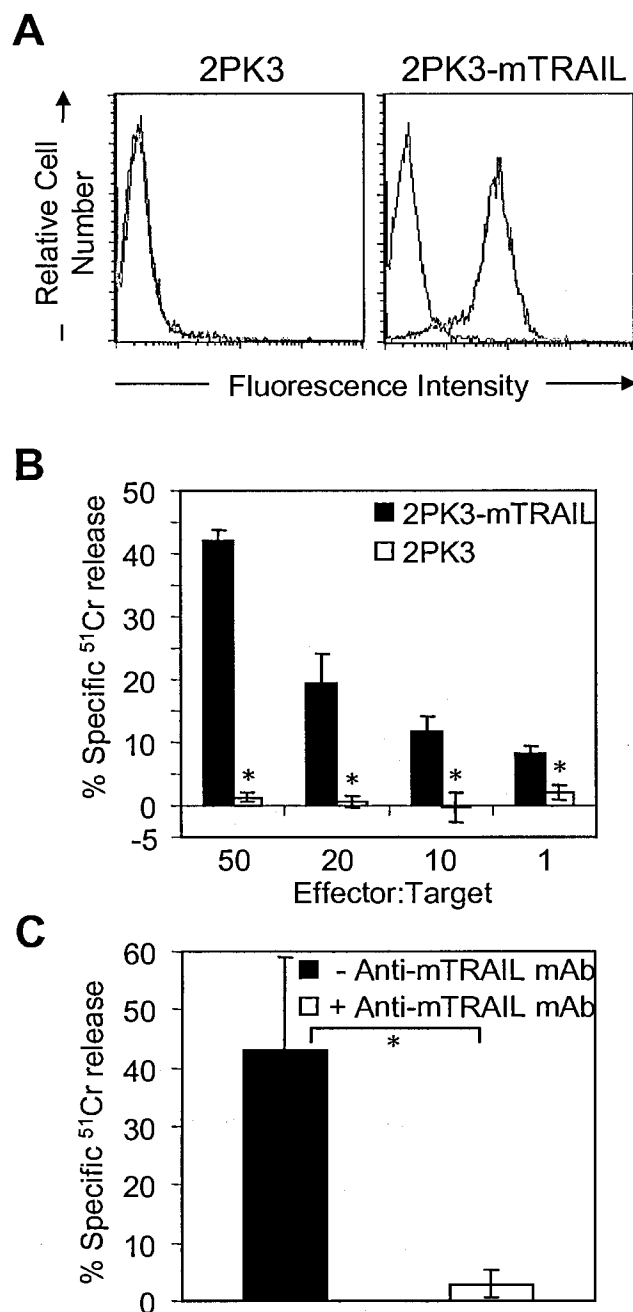


Figure 3.1. Characterization of mouse TRAIL transfectants. (A) Cell surface staining of 2PK3 and 2PK3-mTRAIL transfectant cells with anti-mTRAIL or isotype control mAb. Red histograms represent staining with anti-mTRAIL mAb, black histograms represent staining with isotype matched control mAb. Data are representative of over 10 independent experiments. (B) The cytotoxic activity of 2PK3 and 2PK3-mTRAIL cells against Renca tumor targets was tested in an 18 h ^{51}Cr release assay. Data represent mean \pm SEM of triplicate samples. Data are representative of over 20 independent experiments. (C) Cytotoxic activity of 2PK3-mTRAIL cells was tested in the presence or absence of 1 $\mu\text{g}/\text{ml}$ anti-mTRAIL mAb in an 18 h ^{51}Cr release assay at an effector:target ratio of 50:1. Data represent mean \pm SEM of triplicate samples. Asterisks in (B) indicate that lysis by 2PK3-mTRAIL transfectants is significantly different to lysis by 2PK3 cells, and (C) indicate that lysis in the presence and absence of anti-mTRAIL mAb is significantly different (* $P < 0.03$). Data are representative of 8 independent experiments.

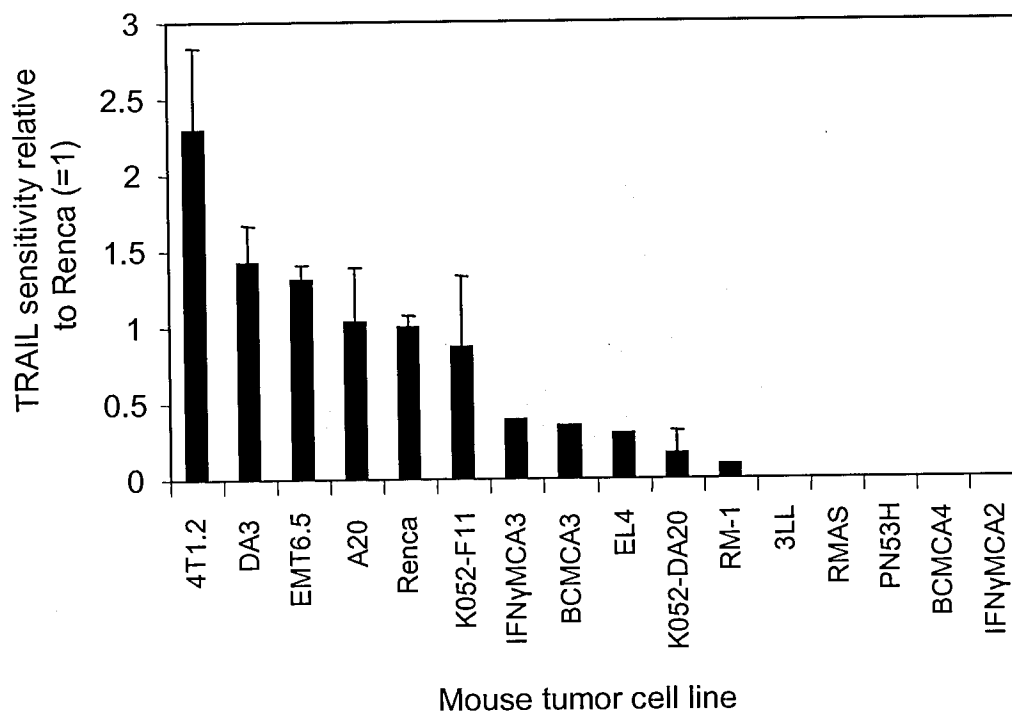


Figure 3.2. Sensitivity of mouse tumor cell lines to TRAIL-mediated cytotoxicity. The cytotoxic activity of 2PK3-mTRAIL transfectant cells against various mouse tumor cell lines was tested in an 18 h ^{51}Cr release assay. Percent specific TRAIL-mediated lysis was determined by subtracting percent specific lysis by 2PK3 parental cells from % specific lysis by 2PK3-mTRAIL transfectant cells. Percent specific TRAIL-mediated lysis for each cell line is shown relative to lysis of Renca (=1) at an effector:target ratio of 50:1, and are representative of those across the effector:target ratio of 100 to 1:1. Data represent mean \pm SEM of 3-20 independent experiments for the 5 most sensitive cell lines, and 1-2 independent experiments for all remaining cell lines.

1983). Following injection into the mammary fat pad, the 4T1.2 tumor spontaneously metastasizes to the lung, liver, LNs, bone, brain and peripheral blood. The 4T1.2 model is one of the best available models of breast cancer metastatic disease and has proven useful for testing various immunotherapies *in vivo* (Lin et al., 1998; Pulaski et al., 2000a; Pulaski and Ostrand-Rosenberg, 1998; Pulaski et al., 2000b; Rakhmilevich et al., 2000). Given that 4T1.2 cells were found to be particularly TRAIL-sensitive, we chose to investigate the role of TRAIL in tumor surveillance using this model.

We first examined the growth of 4T1.2 tumor cells injected into the peritoneum of BALB/c WT, BALB/c TRAIL^{-/-} and BALB/c WT mice treated with a neutralizing anti-mTRAIL mAb. The 4T1.2 tumor was observed to grow as ascites within the peritoneal cavity. At the higher doses of 4T1.2 cells inoculated (1,000 and 10,000 cells i.p.), all mice developed tumor and were sacrificed 15-36 days post-inoculation (Figure 3.3). At lower doses of 4T1.2 cells inoculated (10 and 100 cells i.p.), mice lacking TRAIL were more susceptible to tumor growth (Figure 3.3). Whilst 4/4 BALB/c TRAIL^{-/-} mice and 4/4 BALB/c WT mice treated with anti-mTRAIL mAb succumbed to tumor when inoculated with 100 4T1.2 cells i.p., only 1/4 BALB/c WT mice developed tumor at this dose. Moreover, 1/4 BALB/c TRAIL^{-/-} and 2/4 BALB/c WT mice treated with anti-mTRAIL mAb developed tumor when inoculated with 10 cells i.p., whereas all BALB/c WT mice were tumor free. These results suggested that TRAIL inhibited 4T1.2 tumor growth within the peritoneum.

To ascertain whether TRAIL-mediated inhibition of 4T1.2 i.p. tumor growth was dependent upon pfp or IFN- γ , we assessed tumor growth in BALB/c pfp^{-/-} or BALB/c IFN- γ ^{-/-} mice treated with a neutralizing anti-mTRAIL mAb or PBS. At the higher doses of 4T1.2 cells inoculated (100 and 1000 cells i.p.), all mice developed tumor, except 2/4 BALB/c pfp^{-/-} mice inoculated with 100 4T1.2 cells i.p. that remained tumor free (Figure 3.4). This suggested that mice deficient in pfp are less susceptible to 4T1.2 i.p. tumor growth than mice deficient in IFN- γ . When 10 4T1.2 cells were inoculated i.p., tumor development was equivalent in BALB/c IFN- γ ^{-/-} mice with or without treatment with a neutralizing anti-

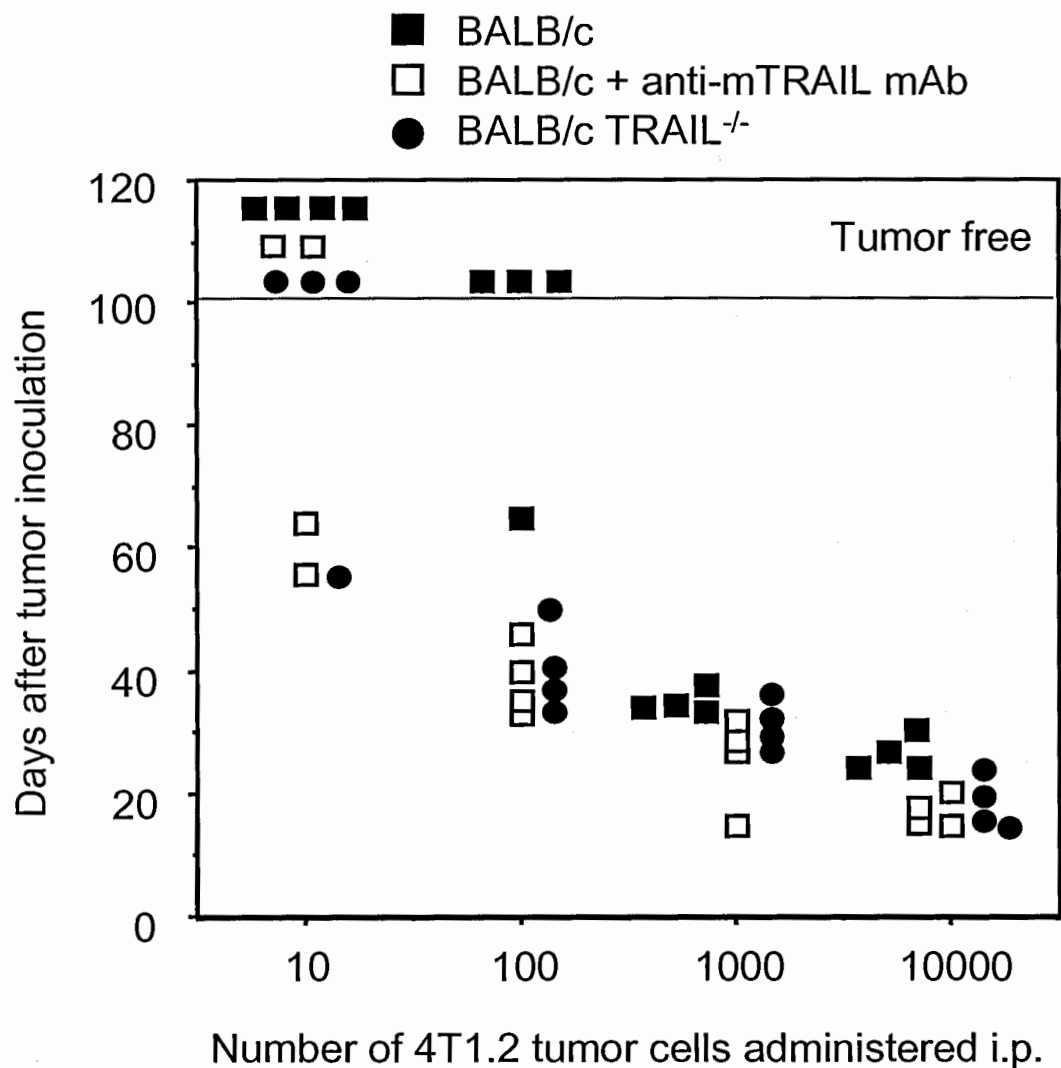


Figure 3.3. TRAIL suppresses 4T1.2 intraperitoneal (i.p.) tumor growth. Groups of female BALB/c or BALB/c TRAIL^{-/-} mice were inoculated on day 0 with 10, 100, 1000 or 10,000 4T1.2 mammary carcinoma cells i.p. Some BALB/c mice were additionally treated with 500 µg anti-mTRAIL mAb i.p. on days 0, 1, 7 and 14. Mice were monitored for ascites development and sacrificed when moribund. Four mice are shown for each group for each tumor dosage, with each mouse represented by an individual symbol. Mice that survived past 100 days were deemed tumor free. Data are representative of 3 independent experiments.

mTRAIL mAb, suggesting that TRAIL-mediated rejection of 4T1.2 i.p. tumor is IFN- γ -dependent (Figure 3.4). In contrast, 3/4 BALB/c pfp^{-/-} mice inoculated with ten 4T1.2 cells i.p. remained tumor free, whilst all BALB/c pfp^{-/-} mice treated with a neutralizing anti-mTRAIL mAb succumbed to tumor (Figure 3.4). This suggested that TRAIL-mediated rejection of 4T1.2 tumor in the peritoneum is pfp-independent.

3.4.3 TRAIL-mediated suppression of 4T1.2 subcutaneous (s.c.) tumor growth

We next assessed primary tumor growth of 4T1.2 tumor cells injected into the mammary gland of BALB/c WT, BALB/c TRAIL^{-/-} and BALB/c WT mice treated with a neutralizing anti-mTRAIL mAb. At the lower doses of 4T1.2 tumor cells injected (5×10^3 and 1×10^4), tumor growth was enhanced in BALB/c TRAIL^{-/-} and BALB/c WT mice treated with a neutralizing anti-mTRAIL mAb, compared to BALB/c WT mice (Figure 3.5A and B). Tumor growth was accelerated even further in mice depleted of NK cells using anti-asialoGM1 Ab (Figure 3.5A and B). At the highest dose of tumor cells inoculated (2.5×10^4), the immune system of the mice was overcome and maximum tumor growth was observed for all groups (Figure 3.5C). Together, these data demonstrated that TRAIL naturally suppressed 4T1.2 tumor growth *in vivo*, and importantly, in the mammary gland itself, a site not previously examined for TRAIL function. Our data additionally indicated that NK cells play an important role in suppression on 4T1.2 tumor growth in the mammary gland.

3.4.4 α -GalCer stimulates TRAIL-mediated cytotoxicity

Administration of IFN- γ has previously been shown to rapidly induce TRAIL expression in the liver and spleen of C57BL/6 IFN- γ ^{-/-} mice (Takeda et al., 2001). As α -GalCer has previously been shown to potently stimulate V α 14 invariant-NKT cells to produce high levels of IFN- γ (Kawano et al., 1997), we next examined the effect of α -GalCer treatment on TRAIL function. Liver MNC isolated from BALB/c WT mice treated with α -GalCer displayed augmented cytotoxicity against 4T1.2 and Renca targets (Figure 3.6A), that was partially

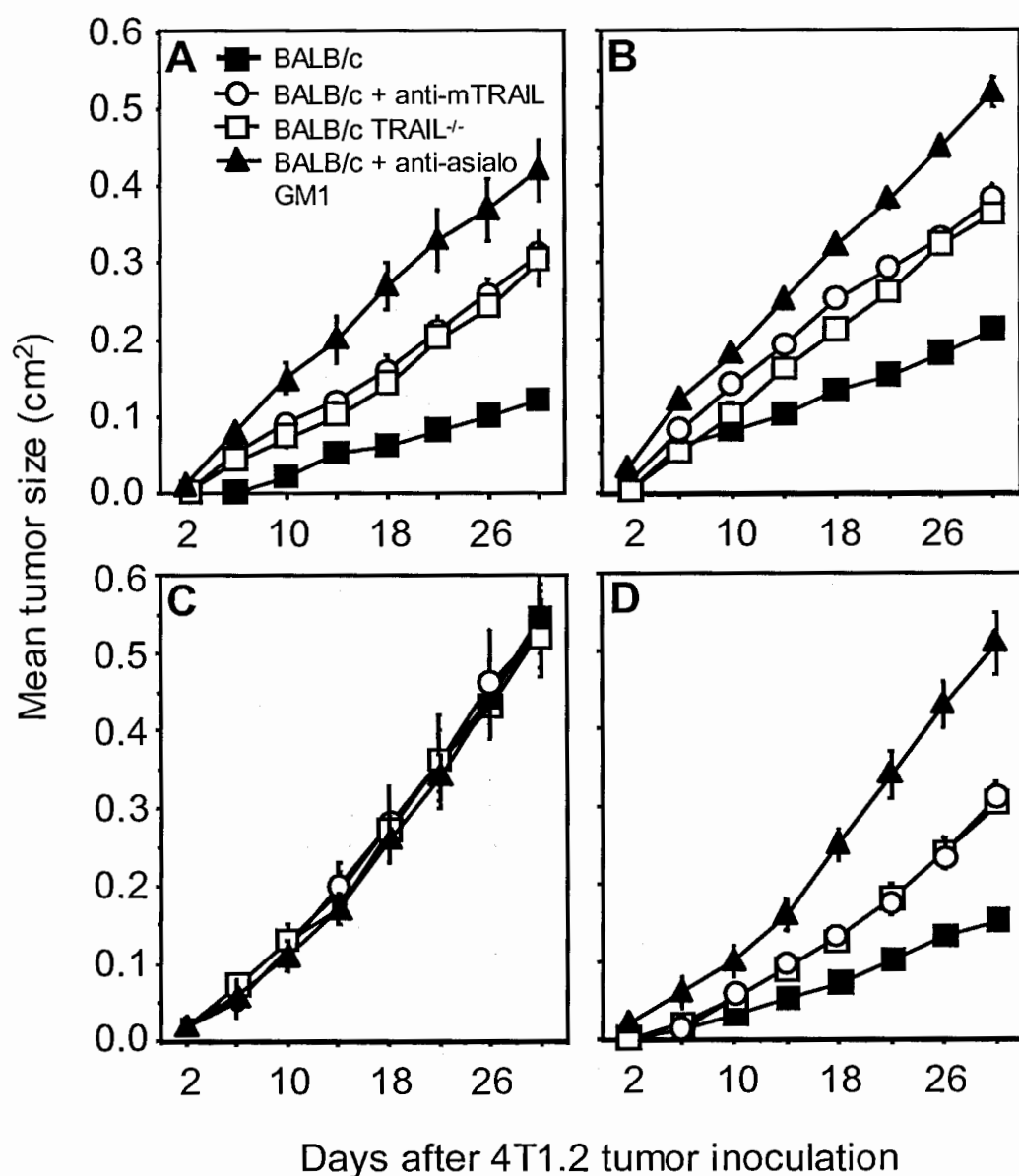


Figure 3.5. TRAIL contributes to the suppression of 4T1.2 primary tumor growth in the mammary fat pad. Groups of female BALB/c or BALB/c TRAIL^{-/-} mice were inoculated on day 0 into the abdominal mammary fat pad with (A) 5×10^3 , (B) 1×10^4 or (C) 2.5×10^4 4T1.2 mammary carcinoma cells. Some BALB/c mice were additionally treated with 200 μ g anti-asialoGM1 Ab on days -1, 0, 7 and 14, or 250 μ g anti-mTRAIL mAb i.p. on days 0, 1, 4, 7, 10, 14 and 21. Tumor size was measured over the course of 30 days. Data represent mean \pm SEM of 5 mice in each group. (D) Groups of female BALB/c or BALB/c TRAIL^{-/-} mice were inoculated on day 0 into the abdominal mammary fat pad with 2.5×10^4 4T1.2 mammary carcinoma cells and were i.p. administered with 2 μ g of α -GalCer on days 0, 4, 8, 12 and 16. Some groups of mice were additionally treated with 200 μ g anti-asialoGM1 Ab on days -1, 0, 7 and 14, or 250 μ g anti-mTRAIL mAb i.p. on days 0, 1, 4, 7, 10, 14 and 21. Tumor size was measured over the course of 30 days. Data represent mean \pm SEM of 5 mice in each group.

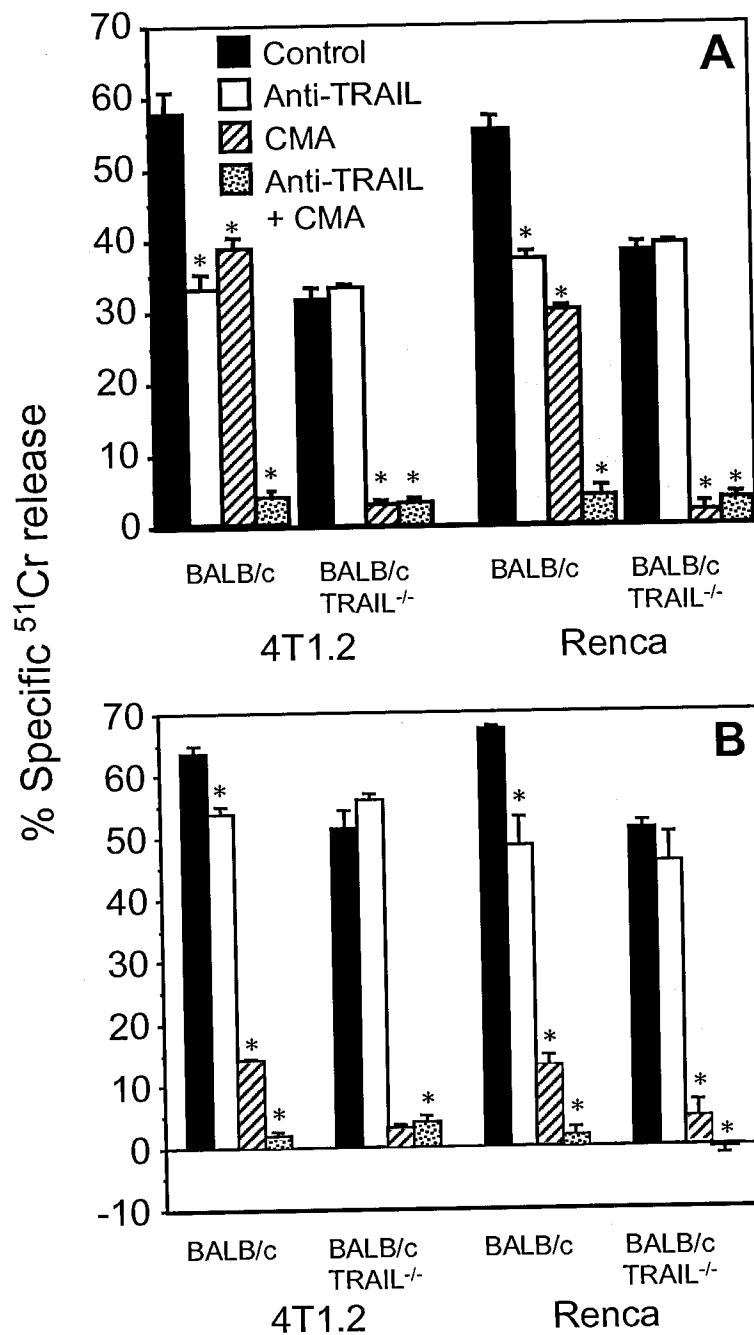


Figure 3.6. α -GalCer treatment augments TRAIL-mediated cytotoxicity in the liver and spleen. Liver and spleen MNC were isolated from adult BALB/c and BALB/c TRAIL^{-/-} mice that had been treated with 2 μ g α -GalCer i.p. on days 0 and 4 and their cytotoxic activities tested against 4T1.2 and Renca target cells on day 5 by an 8 h ⁵¹Cr release assay. Assays were set up at various effector:target ratios, in the presence or absence of 50 nM CMA, 10 μ g/ml anti-mTRAIL mAb, 50 nM CMA plus 10 μ g/ml anti-mTRAIL mAb, or no inhibitor present (control). (A) Liver MNC lysis of 4T1.2 and Renca targets cells is shown at 50:1 effector:target ratio. (B) Spleen MNC lysis of 4T1.2 and Renca target cells is shown at 100:1 effector:target ratio. Data are representative of those across the effector:target ratio of 50 to 5:1 (for liver) and 100 to 5:1 (for spleen). Data represent mean \pm SEM of triplicate samples. Asterisks indicate groups that are significantly different to control (* $P < 0.05$). Data are representative of 2 independent experiments.

abrogated by the addition of neutralizing anti-mTRAIL mAb and completely inhibited by the addition of both anti-mTRAIL mAb and CMA. Liver MNC from BALB/c TRAIL^{-/-} mice treated with α -GalCer displayed increased pfp-mediated cytotoxicity against 4T1.2 and Renca targets, but did not display TRAIL-mediated cytotoxicity (Figure 3.6A). Together, these data indicated that α -GalCer treatment can augment both TRAIL- and pfp-dependent cytotoxicity.

Spleen MNC isolated from BALB/c WT mice treated with α -GalCer also displayed augmented cytotoxicity against 4T1.2 and Renca targets (Figure 3.6B), that was partially abrogated by the addition of neutralizing anti-mTRAIL mAb and completely inhibited by the addition of both anti-mTRAIL mAb and CMA. In contrast, spleen MNC from BALB/c TRAIL^{-/-} mice treated with α -GalCer displayed increased pfp-mediated cytotoxicity against 4T1.2 and Renca targets but did not display TRAIL-mediated cytotoxicity (Figure 3.6B). These data indicated that α -GalCer can stimulate both TRAIL- and pfp-dependent cytotoxicity in spleen MNC.

3.4.5 Therapeutic efficacy of α -GalCer on 4T1.2 tumor growth and metastasis

α -GalCer has previously been shown to enhance NK cell killing of tumor cells when administered to mice (Kitamura et al., 1999). As we had shown that α -GalCer treatment stimulated TRAIL-mediated killing by liver and spleen MNCs, and NK cells and TRAIL suppress 4T1.2 tumor growth in the mammary gland, we next investigated whether TRAIL plays a role in the therapeutic efficacy of α -GalCer *in vivo*. The α -GalCer treatment regime (2 μ g i.p. on days 0, 4, 8, 12 and 16) was chosen based on previous efficacy studies in the Renca tumor model (Smyth et al., 2001). Whilst α -GalCer treatment significantly inhibited the growth of 4T1.2 cells in the mammary gland (Figure 3.5D), α -GalCer had no effect in mice depleted of NK cells, and was only partially effective in BALB/c TRAIL^{-/-} mice and BALB/c WT mice treated with a neutralizing anti-mTRAIL mAb (Figure 3.5D). These findings indicated that TRAIL played a significant

role in the NK cell-mediated therapeutic efficacy of α -GalCer against 4T1.2 tumor growth in the mammary gland.

Following injection into the mammary gland, the 4T1.2 tumor spontaneously metastasizes to a number of sites, including the lung and liver. Given that α -GalCer significantly inhibited 4T1.2 primary tumor growth in the mammary gland, we examined the effect of α -GalCer on 4T1.2 lung and liver metastasis. α -GalCer significantly reduced both lung and liver metastasis in BALB/c WT mice ($P < 0.01$) (Figure 3.7). Moreover, the anti-metastatic effect of α -GalCer was completely abrogated in mice depleted of NK cells, indicating a critical contribution by NK cells (Figure 3.7). In the lung, the anti-metastatic effect of α -GalCer was TRAIL-independent (Figure 3.7). In contrast, α -GalCer was only partially effective in the liver of BALB/c TRAIL^{-/-} mice and BALB/c WT mice treated with a neutralizing anti-mTRAIL mAb (Figure 3.7), suggesting that TRAIL contributes to the anti-metastatic effect of α -GalCer in the liver.

3.4.6 TRAIL-mediated suppression of DA3 s.c. tumor growth

As DA3 tumor cells were found to be particularly TRAIL-sensitive *in vitro*, we investigated the role of TRAIL in tumor surveillance using the DA3 model of breast cancer metastasis. We first assessed s.c. growth of DA3 tumor cells injected into BALB/c WT and BALB/c TRAIL^{-/-} mice. DA3 tumor growth was accelerated in BALB/c TRAIL^{-/-} compared to BALB/c WT mice at all doses tested (Figure 3.8). This indicated the potency of TRAIL in eradication of a bolus of tumor cells transplanted at a peripheral site.

3.4.7 Generation of DA3-H60 infectants

Previously, NK and CD8⁺ T cells have been identified to reject NKG2D ligand expressing tumor cells *in vivo* (Cerwenka et al., 2001; Diefenbach et al., 2001). Recent studies in our laboratory have demonstrated that NKG2D can stimulate pfp-mediated rejection of NKG2D ligand expressing tumor cells *in vivo* (Hayakawa et al., 2002). As the contribution of the TNF-superfamily to NKG2D-stimulated tumor rejection is unknown, we chose to investigate the role of TRAIL

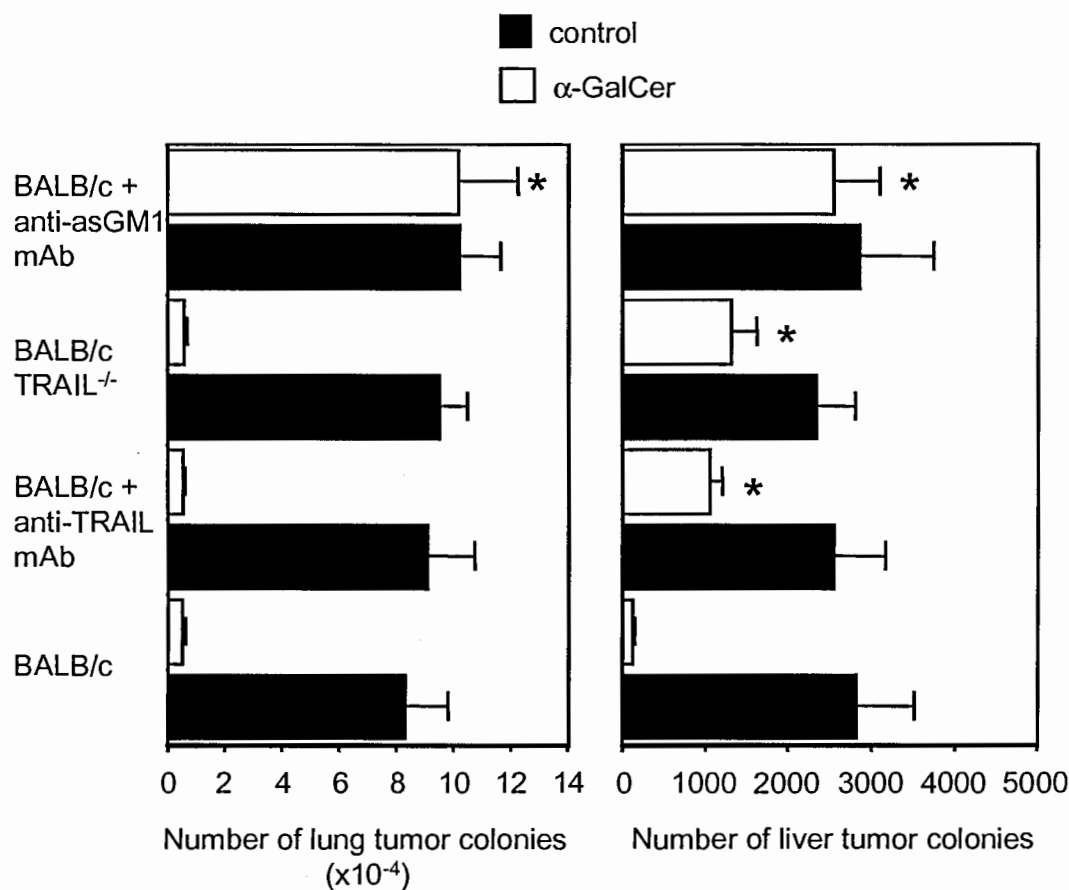


Figure 3.7. TRAIL contributes to the suppression of 4T1.2 spontaneous metastasis. Groups of female BALB/c or BALB/c TRAIL^{-/-} mice were inoculated on day 0 into the abdominal mammary fat pad with 2.5×10^4 4T1.2 mammary carcinoma cells. Some mice were additionally administered i.p. with 2 μ g of α -GalCer on days 0, 4, 8, 12 and 16, and/or 200 μ g anti-asialoGM1 Ab on days -1, 0, 7 and 14, or 250 μ g anti-mTRAIL mAb i.p. on days 0, 1, 4, 7, 10, 14 and 21. Mice were sacrificed at 30 days, their lungs and livers harvested and metastases counted by colony assay. Data represent mean \pm SEM of 5 mice in each group, with the significance compared with α -GalCer-treated BALB/c WT mice (* $P < 0.01$). α -GalCer was also statistically effective alone compared with no treatment in the livers and lungs of BALB/c WT mice (* $P < 0.01$).

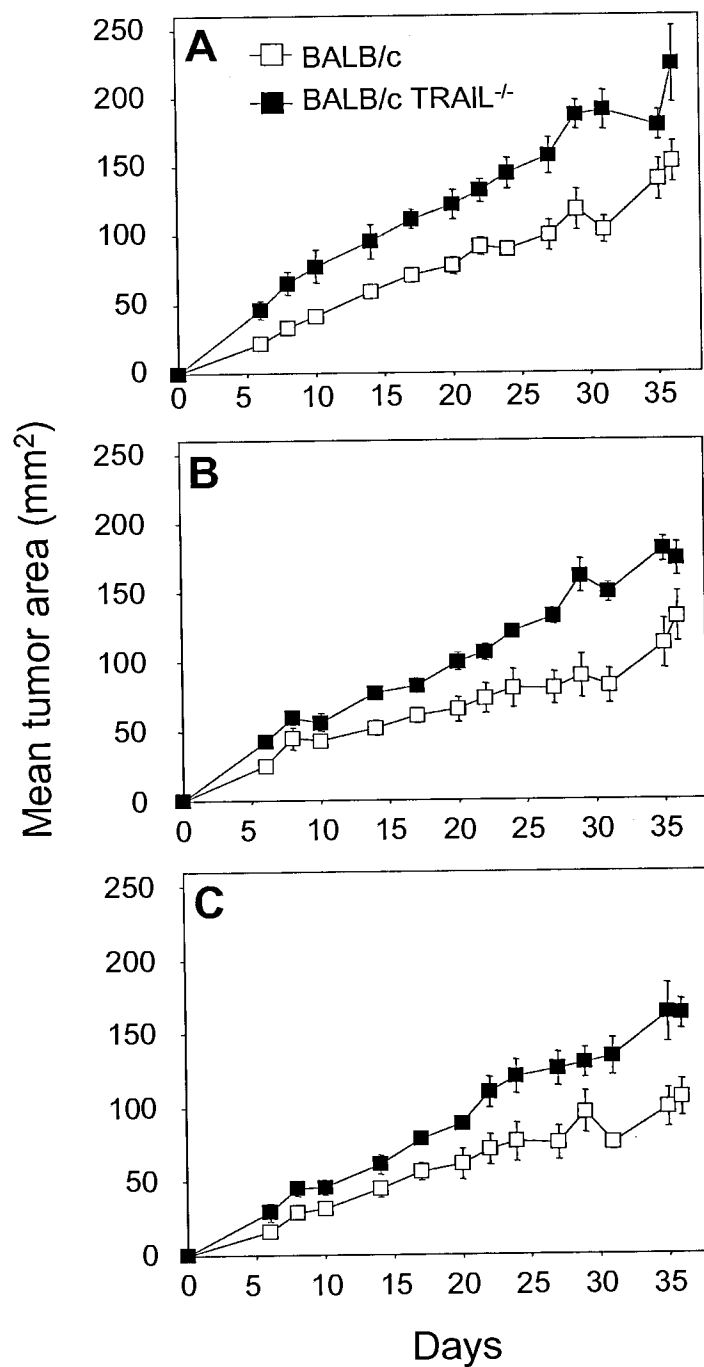


Figure 3.8. TRAIL suppresses DA3 s.c. growth. Groups of female BALB/c or BALB/c TRAIL-deficient mice were inoculated with (A) 3×10^6 (B) 1.5×10^6 (C) 5×10^5 DA3 mammary carcinoma cells s.c. Tumor size was measured over the course of 36 days. Data represent mean \pm SEM of 5 mice in each group. Data are representative of 4 independent experiments for each dose inoculated.

in the primary immunity generated by the DA3 tumor cells expressing the NKG2D ligand H60. Initially, DA3-MSCV (vector alone control) and DA3-H60 infectants were produced by retroviral transduction. H60 expression was examined on these cells using a NKG2D tetramer that specifically binds to cells that express NKG2D ligand (Figure 3.9). A high level of NKG2D ligand expression was detected on DA3-H60 infectant cells and a low level on DA3-MSCV control cells (Figure 3.9). To verify the low level of NKG2D ligand expression detected in DA3 parental cells, mRNA from DA3 parental cells was analyzed by RT-PCR. A low intensity H60 amplification product 740bp in size was detected in DA3 cells (Figure 3.10), confirming a low level of H60 expression in these cells. MHC class I (H-2D^d) and TRAIL receptor (DR5) expression were additionally examined on DA3-MSCV and DA3-H60 cells and found to be equivalent (Figure 3.10). This ensured that any detectable differences in tumor rejection of DA3-MSCV and DA3-H60 cells *in vivo* were due to H60 expression rather than the ability of these cells to evade detection by NK cells or their susceptibility to TRAIL-mediated killing.

3.4.8 H60-mediated inhibition of DA3 s.c. tumor growth and metastasis

To investigate whether H60 expressed on DA3 cells could induce tumor rejection, BALB/c WT mice were injected s.c. with either DA3-H60 or DA3-MSCV control cells. The DA3 mouse mammary carcinoma spontaneously metastasizes to the lung when injected s.c. in mice. DA3-H60 cells were rejected more avidly than DA3-MSCV control cells, confirming that H60 expression induced primary tumor rejection (Figure 3.11A). Spontaneous metastasis to the lung (Figure 3.11B) was also decreased in mice injected with DA3-H60 compared to DA3-MSCV control cells, illustrating that H60 can potentially stimulate NK cell-mediated tumor rejection *in vivo*.

3.4.9 H60-mediated inhibition of spontaneous DA3 lung metastasis

DA3 tumor cells injected i.v. into mice spontaneously metastasize to the lung. We next investigated the relative contribution of pfp and TRAIL in the rejection of spontaneous DA3-H60 lung metastasis *in vivo*. BALB/c WT, BALB/c pfp^{-/-},

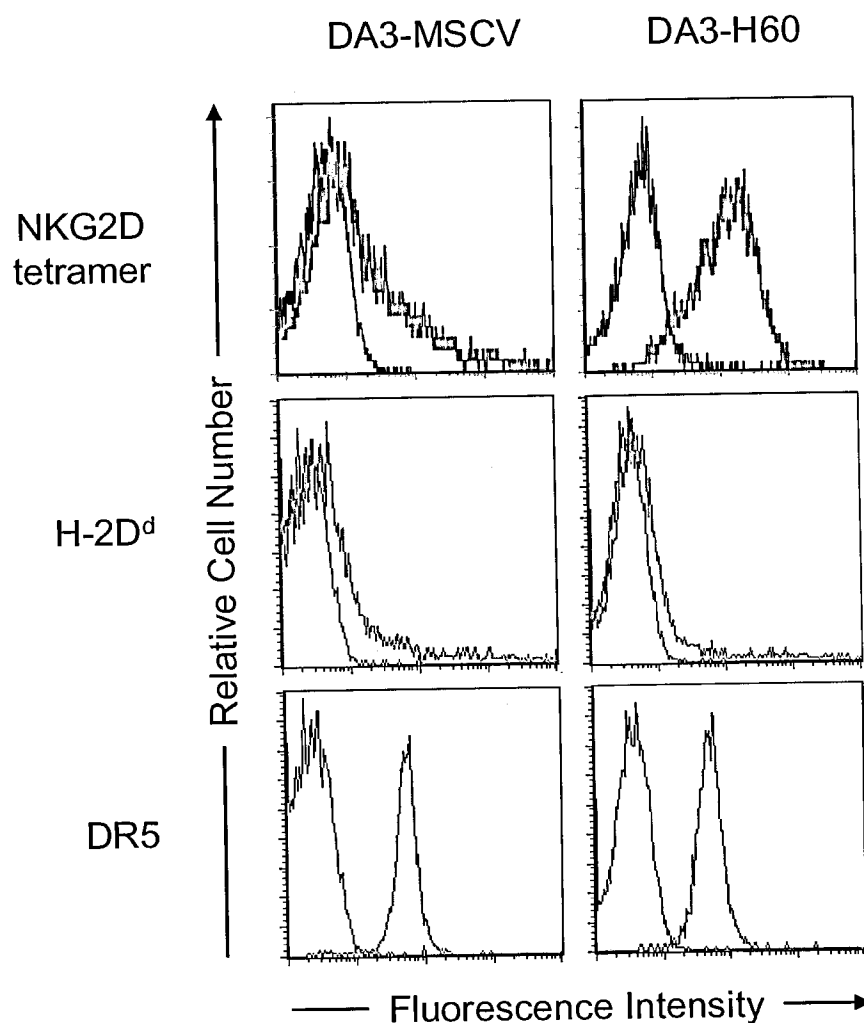


Figure 3.9. Characterization of DA3-H60 retroviral infectants. Cell surface staining of DA3-MSCV and DA3-MSCV-H60 infectants with NKG2D tetramer or control T22-tetramer, anti-H-2D^d mAb, anti-DR5 mAb or isotype control mAb. Red histograms represent staining with NKG2D tetramer, anti-H-2D^d mAb or anti-DR5 mAb as indicated. Black histograms represent staining control T22-tetramer, or with isotype matched control mAb. Data are representative of 5 independent experiments.

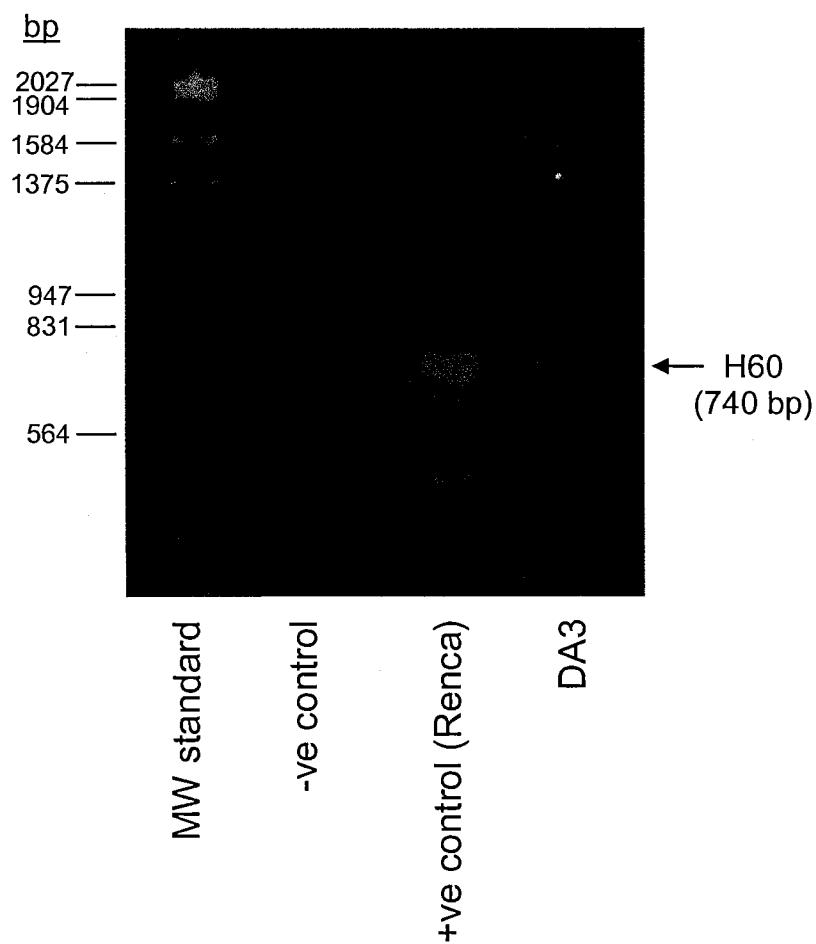


Figure 3.10. Detection of H60 transcript in DA3 cells. RNA was isolated from DA3 cells (or Renca as a positive control), and first-strand cDNA synthesis was performed, followed by PCR amplification using specific primers to H60. As a negative control, the RT product was omitted from the PCR reaction. Samples were separated on a 2% agarose gel, and DNA visualized by ethidium bromide staining under UV illumination. An H60 amplification product of 740bp is shown for Renca and DA3 cells.

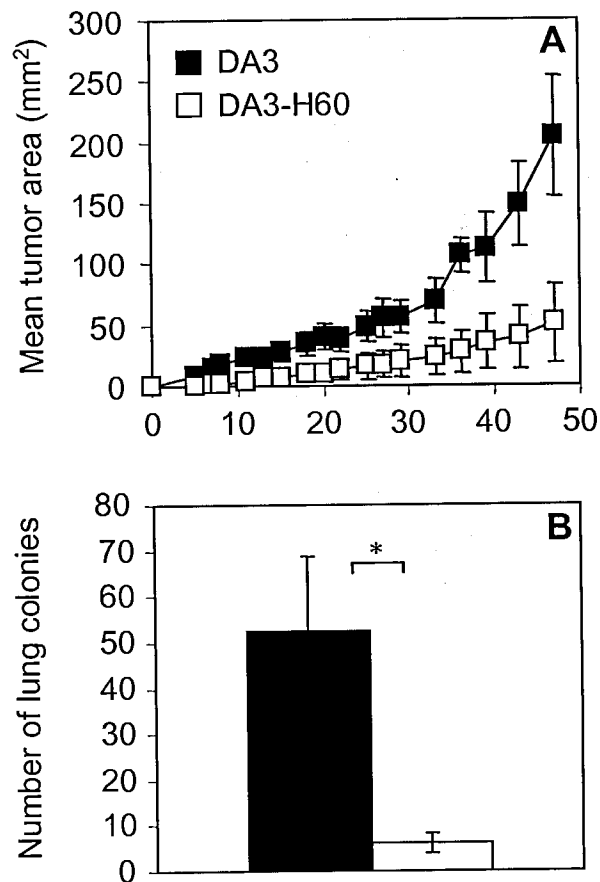


Figure 3.11. H60 expression inhibits DA3 s.c. tumor growth and metastasis to the lung. Groups of female BALB/c mice were inoculated with 5×10^5 DA3-MSCV or DA3-MSCV-H60 mammary carcinoma cells s.c. (A) S.c. tumor size was measured over the course of 47 days. Mice were sacrificed after 47 days and (B) lungs harvested, stained with Bouin's solution and metastases counted. Data represent mean \pm SEM (n= 6/group). Asterisk indicates that numbers of lung colonies are significantly different in mice injected with DA3 and DA3-H60 (* P < 0.02).

BALB/c TRAIL^{-/-} and BALB/c pfp^{-/-} TRAIL^{-/-} mice were injected with DA3-MSCV or DA3-H60 cells i.v. and lung metastases quantified after 14 days. At all doses of tumor tested, H60 expression significantly reduced the number of lung metastasis in BALB/c WT mice, and completely suppressed metastasis at the lowest dose of tumor cells inoculated (5×10^4) (Figure 3.12). H60 expression also reduced numbers of lung metastasis in BALB/c pfp^{-/-} and BALB TRAIL^{-/-} mice, but had no effect on the number of lung metastasis in BALB/c pfp^{-/-} TRAIL^{-/-} mice (Figure 3.12). These data suggested that NKG2D stimulated pfp- and TRAIL-mediated rejection of DA3-H60 lung metastasis, and pfp played a dominant role in this process.

■ WT + DA3 ▨ WT + DA3-H60
 ▩ Pfp^{-/-} + DA3 ▨ Pfp^{-/-} + DA3-H60
 ■ TRAIL^{-/-} + DA3 ▨ TRAIL^{-/-} + DA3-H60
 ▩ Pfp^{-/-}TRAIL^{-/-} + DA3 ▨ Pfp^{-/-}TRAIL^{-/-} + DA3-H60

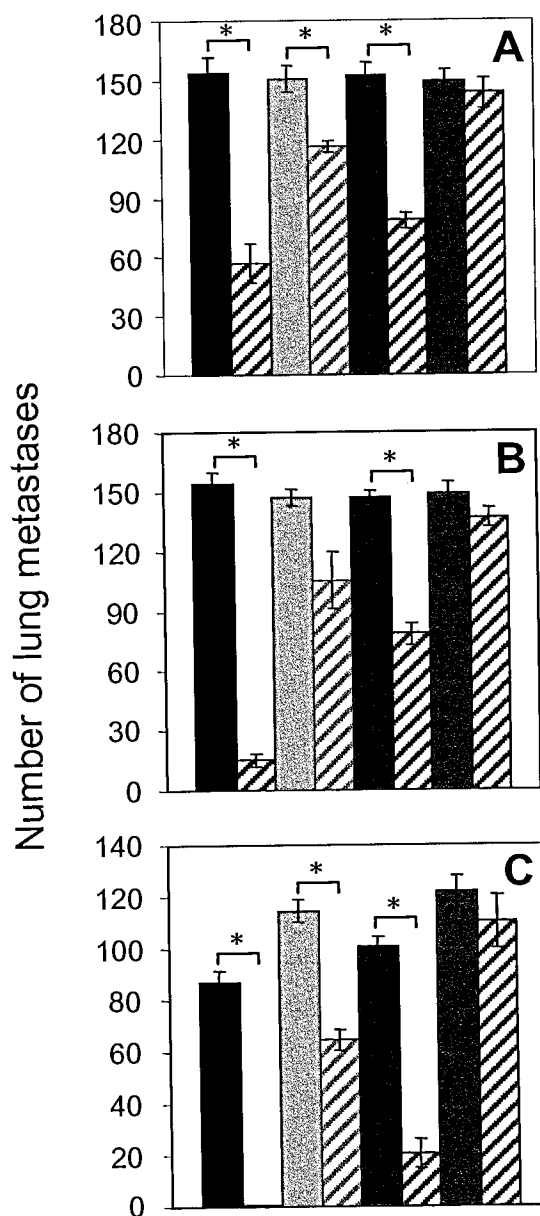


Figure 3.12. NKG2D-mediated inhibition of DA3 lung metastases is largely pfp-dependent. Groups of female BALB/c, BALB/c pfp^{-/-}, BALB/c TRAIL^{-/-} and BALB/c pfp^{-/-} TRAIL^{-/-} mice were inoculated with (A) 5 x 10⁵, (B) 2 x 10⁵, or (C) 5 x 10⁴ DA3-MSCV or DA3-MSCV-H60 mammary carcinoma cells i.v. Mice were sacrificed at 14 days, their lungs harvested, stained with Bouin's solution and metastases counted. Data represent mean \pm SEM (n= 5/group). Asterisks and parentheses indicate groups that are significantly different (* P < 0.008).

3.5 Discussion

Despite the breadth of knowledge regarding rTRAIL, less is known about the role TRAIL plays in natural tumor immunity. This knowledge is fundamental if the TRAIL pathway is to be used safely and with efficacy in the clinic. In the present study, we identified a number of TRAIL-sensitive mouse tumor models that were useful to dissect the role of TRAIL in tumor surveillance. In particular, the TRAIL-sensitive 4T1.2 and DA3 mouse mammary carcinomas that closely approximate human breast cancer metastatic disease will be useful in the future to assess TRAIL function in a number of sites including the brain, bone, LN, peripheral blood, lung and liver. Interestingly, we observed that a large proportion of BALB/c-derived tumor cells lines were TRAIL-sensitive, whilst the majority of C57BL/6-derived tumor cell lines were resistant to TRAIL-mediated apoptosis. Whilst the differences in sensitivities are quite striking, these tumor cell lines have undergone extensive *in vitro* passage in tissue culture, and the differences may just be random. In Chapter 2 we reported decreased constitutive TRAIL expression on NK cells in BALB/c compared to C57BL/6 mice. Moreover, TRAIL-sensitive tumors have been reported to preferentially emerge in anti-mTRAIL mAb-treated mice (Takeda et al., 2002). From these findings, we speculate that decreased TRAIL expression in BALB/c mice might predispose them to the development of TRAIL-sensitive tumors, that are typically eliminated in C57BL/6 mice.

During this candidature, our laboratory reported that endogenous TRAIL expressed on liver NK cells was at least partially responsible for the suppression of TRAIL-sensitive L929, LB27.4 and Renca experimental liver metastasis *in vivo* (Smyth et al., 2001; Takeda et al., 2001). Our study is the first to demonstrate TRAIL-mediated suppression of tumor growth in a number of unique sites including the peritoneum, mammary gland and lung. Whether TRAIL is constitutively expressed on cells within these unique sites is of great interest, particularly in the mammary gland, a common site of human neoplasia. In addition, it would be interesting to examine whether TRAIL⁺ NK cells migrate into these sites, or whether NK cells become activated and express TRAIL within

these sites in response to tumor growth. Future experiments using the 4T1.2 breast cancer metastasis model that metastasizes to sites including peripheral blood, bone and brain, and the TRAIL gene-targeted mice described in this thesis will be useful to assess TRAIL function in novel sites.

Although a number of studies have evaluated the therapeutic efficacy of α -GalCer in the suppression of tumor metastases, few studies have assessed the ability of α -GalCer to control primary tumor growth. Suppression of tumor growth at the primary site might be useful to eliminate residual cancer cells that have failed to be cleared from the primary tumor site post-surgery. Our study has indicated that α -GalCer can stimulate NK cells and TRAIL to control primary tumor growth in the mammary gland, suggesting that α -GalCer-treatment might be beneficial for the treatment of patients with breast cancer. Using the 4T1.2 model, we additionally showed that TRAIL contributed to the anti-metastatic effect of α -GalCer in the liver, but not in the lung. Further studies in our laboratory using the Renca renal carcinoma tumor model similarly identified a contribution by TRAIL to the anti-metastatic effect of α -GalCer in the liver, but not in the lung (Smyth et al., 2001). In agreement with our findings, administration of α -GalCer *in vivo* stimulated TRAIL expression and cytotoxic activity in the liver NK cells, but not in lung NK cells (Smyth et al., 2001). Importantly, although our studies have indicated that α -GalCer can limit the growth and establishment of tumors *in vivo*, future studies are required to determine whether α -GalCer treatment can suppress the growth of established tumors *in vivo*.

During this candidature, a number of additional studies undertaken in our laboratory and others have greatly contributed to our knowledge of the role TRAIL plays in anti-tumor immunity. Importantly, the TRAIL gene-targeted mice characterized in the current study were used to identify a role for TRAIL in natural host protection from MCA-induced fibrosarcoma development (Cretney et al., 2002). Moreover, TRAIL-sensitive MCA-induced tumors were identified to preferentially emerge in anti-mTRAIL mAb-treated, NK cell-depleted, and IFN- γ ^{-/-} mice, indicating that TRAIL plays an important role in NK cell- and IFN-

γ -mediated immune surveillance (Takeda et al., 2002). A role for TRAIL in natural protection from spontaneous tumor development in C57BL/6 p53^{+/-} mice was also identified (Takeda et al., 2002). In a separate study, transfection of TRAIL-sensitive R331 renal carcinoma cells with cFLIP increased the number of liver metastasis in mice inoculated with this tumor, illustrating the importance of TRAIL-mediated apoptosis in control of tumor growth *in vivo* (Seki et al., 2003). TRAIL-mediated necrosis and apoptosis of tumor cells were also described, in studies using rTRAIL adenovirus (Griffith et al., 2000; Kemp et al., 2003b). The same group of researchers also reported that specific CpG-containing oligodeoxynucleotides can induce TRAIL expression and anti-tumor activity in human B cells and macrophages, implicating these cells in the suppression of tumor growth (Kemp et al., 2003a; Kemp et al., 2004). IL-12 administration was additionally reported to induce TRAIL expression and cytotoxic activity on lung and liver NK cells, and the anti-metastatic activity of IL-12 in these organs was partially mediated by TRAIL (Smyth et al., 2001). Together these studies, combined with those reported herein, reveal an important and general role for TRAIL in tumor surveillance.

NKG2D ligand-expressing tumor cells are rejected by NK and CD8⁺ T cells *in vivo* (Cerwenka et al., 2001; Diefenbach et al., 2001). Recent studies in our laboratory have demonstrated the importance of pfp-mediated cytotoxicity in NKG2D-mediated tumor rejection *in vivo* (Hayakawa et al., 2002). Given that we had identified TRAIL to be important in NK cell-mediated tumor surveillance *in vivo*, we hypothesized that TRAIL might play a role in NKG2D-mediated tumor rejection by NK cells. The previous study that identified pfp to be important in NKG2D-mediated tumor rejection *in vivo* utilized TRAIL-resistant RMA-S lymphoma cells. In our study, we used TRAIL-sensitive DA3-H60 infectant cells to demonstrate that TRAIL plays a minor role in NKG2D-mediated rejection of lung metastasis. Given that the anti-tumor activity of endogenous TRAIL is greater in the liver compared to the lung, it would be interesting to evaluate the contribution of TRAIL to NKG2D-mediated rejection of liver metastases.

Together our studies using anti-mTRAIL mAb and TRAIL gene-targeted mice have supported a direct role for TRAIL in suppression of 4T1.2 and DA3 tumor initiation and metastasis, and the therapeutic efficacy of α -GalCer in the liver and mammary gland. Our results additionally indicated that TRAIL played a minor role in NKG2D-mediated rejection of H60 expressing tumor cells *in vivo*. Increasing our knowledge of the role of TRAIL in natural tumor immunity will allow more effective design of future clinical trials using rTRAIL to treat cancer. Manipulating TRAIL activity using novel compounds such as α -GalCer might also prove effective in the suppression of tumor initiation and metastasis *in vivo*. Importantly, our studies might allow for advance predictions to be made on the relative effectiveness of various TRAIL therapies (eg. adjuvants and cytokine therapies) based on the site of the primary tumor, the site of tissue metastases and the inherent sensitivity of the tumor to TRAIL-mediated apoptosis.

CHAPTER FOUR

THE ROLE OF TRAIL AND TNF IN IMMUNOREGULATION

4.1 Abstract

In Chapter 3, TRAIL was demonstrated to suppress tumor initiation, growth and metastasis. In addition to an anti-tumor function, some members of the TNF superfamily, such as FasL, play a major role in homeostasis of the peripheral immune system. *Gld* mice that carry a loss of function mutation in the *FasL* gene, develop splenomegaly, lymphadenopathy, hypergammaglobulinemia, strain-dependent autoimmune disease and succumb to premature death as a consequence of impaired peripheral deletion of activated lymphocytes. *Gld* mice have also been reported to develop plasmacytomas with age, suggesting a role for the Fas/FasL pathway in the inhibition of tumor development. Previously, we have shown that mice deficient for both FasL and TNF have a substantially milder *gld* phenotype with regard to mortality, lymphoaccumulation, hypergammaglobulinemia and GC formation. Herein, we investigate further the effect of TRAIL deficiency on the *gld* phenotype by generating mice deficient for both FasL and TRAIL. Plasmacytoma development in aged C57BL/6 *gld* TNF^{-/-} mice was also examined. We report that absence of TRAIL has no discernable effect on the *gld* phenotype and that TNF is not required for plasmacytoma development in aged C57BL/6 *gld* mice.

4.2 Introduction

Homeostasis of the immune system is controlled at several stages of lymphocyte development and involves both activation/proliferation and cell death. The importance of the Fas/FasL pathway in lymphocyte homeostasis is clearly demonstrated in mice carrying loss of function mutations in the *Fas* (*lpr*, lymphoproliferation) or *FasL* (*gld*, generalized lymphoproliferative disease) genes (Adachi et al., 1993; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Watson et al., 1992) or human ALPS patients who harbor Fas/FasL deficiencies (Fisher et al., 1995; Rieux-Laucat et al., 1995; Drappa et al., 1996).

Gld and *lpr* mice develop lymphadenopathy, splenomegaly, hypergammaglobulinemia, strain-dependent autoimmune disease and succumb to premature death (Cohen and Eisenberg, 1991; Izui et al., 1984; Roths et al., 1984; Theofilopoulos and Dixon, 1981). Lymphoproliferative disease in ALPS patients, and *lpr* and *gld* mice, is characterized by an accumulation of B220⁺TCR $\alpha\beta$ ⁺CD4⁻CD8⁻ double negative (B220⁺ DN) T cells in LNs and spleen (Cohen and Eisenberg, 1991; Rieux-Laucat et al., 1999; Sneller et al., 1997) that are typically eliminated by Fas/FasL interactions.

Previously we examined the effect of TNF deficiency on the *gld* phenotype by generating mice doubly deficient in FasL and TNF (Korner et al., 2000). Lymphoaccumulation was markedly reduced in C57BL/6 *gld* TNF^{-/-} compared to C57BL/6 *gld* mice (Figure 4.1). From the age of 16 weeks, the weight of secondary lymphoid organs [mesenteric LN (mLN), pLN and spleen] dramatically increased in C57BL/6 *gld* mice, but only slightly increased in C57BL/6 *gld* TNF^{-/-} mice compared to C57BL/6 and C57BL/6 TNF^{-/-} controls (Figure 4.1). As hypergammaglobulinemia and circulating autoantibodies are hallmark features of the *gld* phenotype (Cohen and Eisenberg, 1991), the concentration of serum IgG, anti-IgG autoantibody and anti-dsDNA autoantibody were also compared. From 16 weeks of age, the concentration of serum IgG was 20-65% lower in C57BL/6 *gld* TNF^{-/-} compared to C57BL/6 *gld* mice (Figure 4.2). However, serum IgG levels in C57BL/6 *gld* TNF^{-/-} mice were still elevated

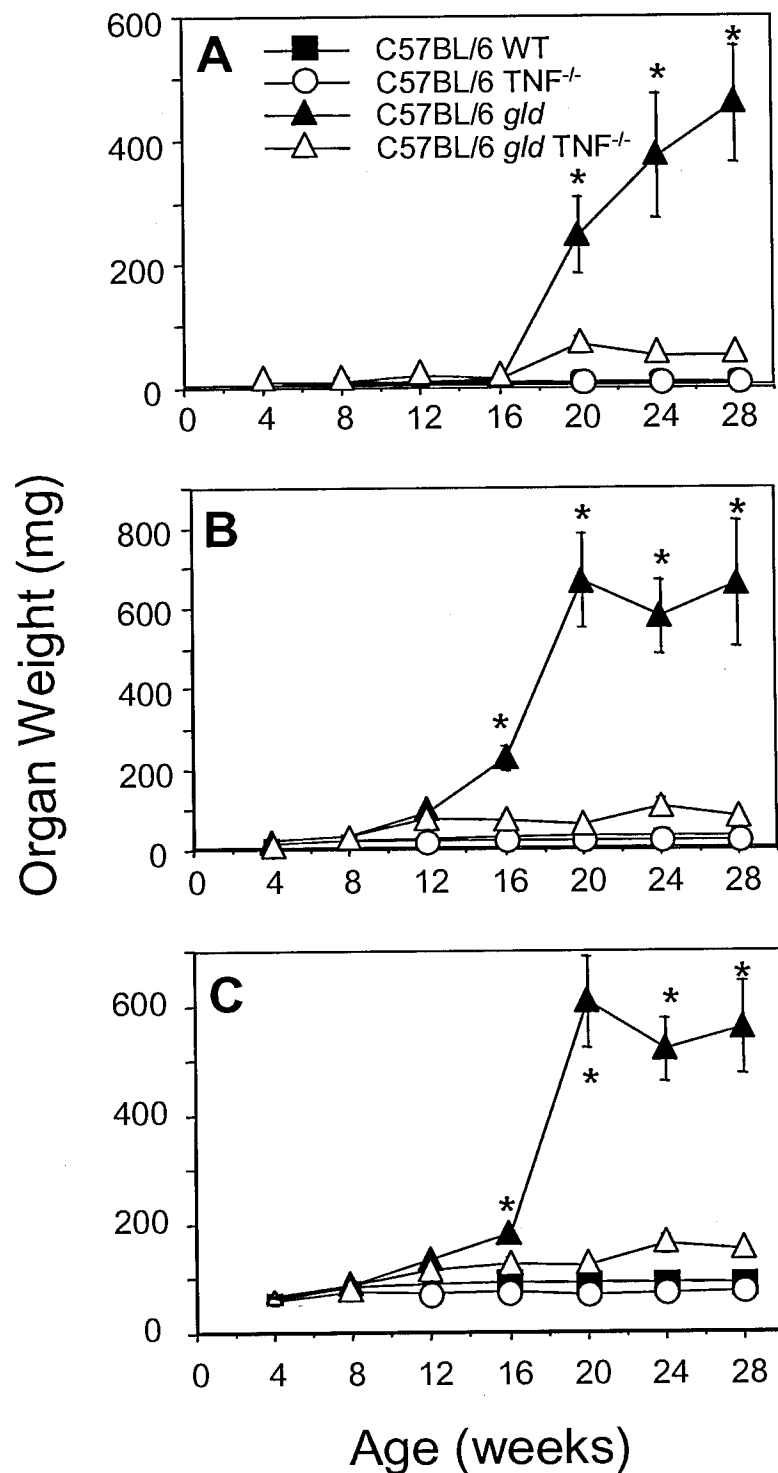


Figure 4.1. Reduced lymphoaccumulation in mice doubly deficient for FasL and TNF. (A) mLN (2 randomly chosen), (B) pLN (two axillary, two cervical and two inguinal) and (C) spleen weights are shown in milligrams (mg) for C57BL/6, C57BL/6 TNF^{-/-}, C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice at 4, 8, 12, 16, 20, 24 and 28 weeks of age. Data represent mean \pm SEM of six mice at each time point, for each genotype. Asterisks indicate the groups of C57BL/6 *gld* mice that are significantly different from C57BL/6 *gld* TNF^{-/-} mice (* $P < 0.04$).

Please note: Data shown in Figure 4.1 was generated by Erika Cretney prior to undertaking PhD candidature (Korner et al., 2000).

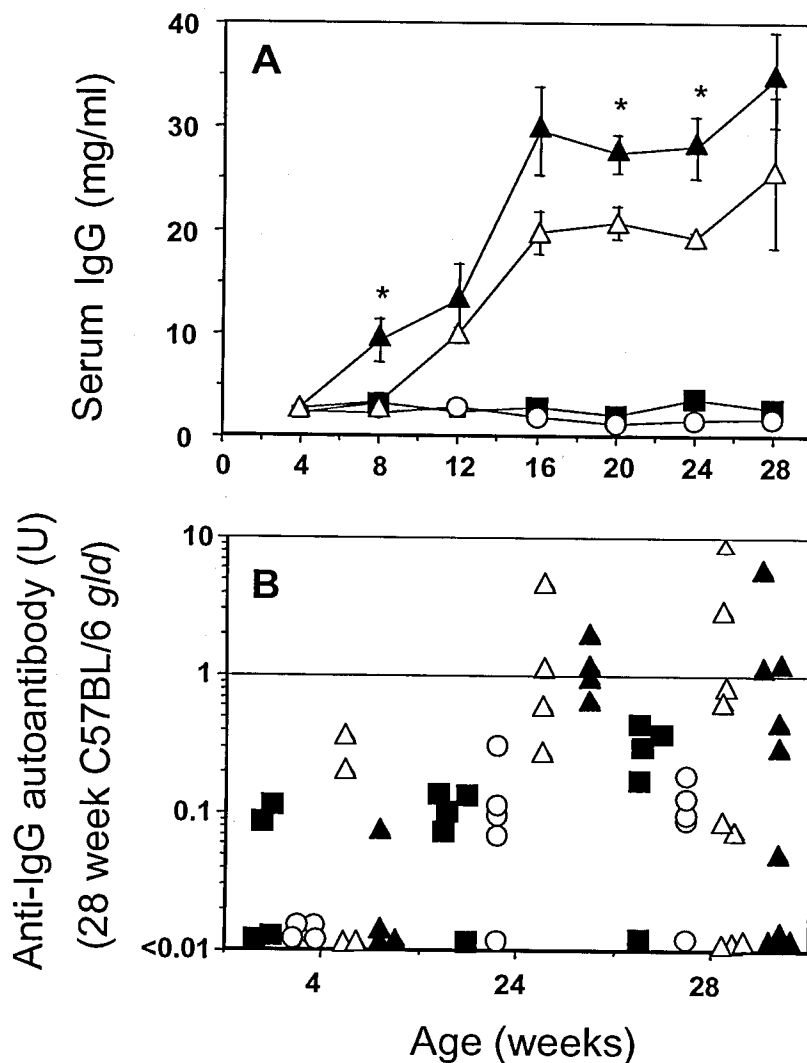


Figure 4.2. Reduced serum IgG but equivalent autoantibody production in mice doubly deficient for FasL and TNF. Serum was isolated from C57BL/6 (■), C57BL/6 TNF^{-/-} (○), C57BL/6 *gld* (▲) and C57BL/6 *gld* TNF^{-/-} (△) mice at 4, 8, 12, 16, 20, 24 and 28 weeks of age and tested by ELISA for (A) total concentration of IgG, (B) anti-IgG autoantibody (IgM, B). The concentration of anti-IgG autoantibodies are shown relative to that observed for 28 week C57BL/6 *gld* mice (1 Unit was defined as the mean of absorbance at 405nm for 28 week C57BL/6 *gld* mice). Mean \pm SEM for (A) were calculated from the analysis of four to six mice at each time point, for each genotype. In (B), four to nine mice are shown at each time point, for each genotype, with each mouse represented by an individual symbol. Asterisks indicate groups of C57BL/6 *gld* mice that are significantly different from C57BL/6 *gld* TNF^{-/-} mice (* P < 0.05).

Please note: Data shown in Figure 4.2 was generated by Erika Cretney prior to undertaking PhD candidature (Korner et al., 2000).

10-20-fold compared with control C57BL/6 and C57BL/6 TNF^{-/-} mice, suggesting that TNF only plays a minor role in hypergammaglobulinemia associated with *gld* (Figure 4.2A). Analysis of serum anti-dsDNA (data not shown), and anti-IgG (Figure 4.2B) autoantibody levels in C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice failed to reveal a role for TNF in autoantibody production, although the qualitative effect of these antibodies was not examined. To further characterize the role of TNF in contributing to the *gld* phenotype, B220⁺ DN T cell accumulation was examined. Although proportions of B220⁺ DN T cells were equivalent in C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} spleen, total numbers of these cells were increased in C57BL/6 *gld* mice, due to increased spleen size in these mice. In contrast, proportions of B220⁺ DN T cells were decreased in the blood of C57BL/6 *gld* TNF^{-/-} compared to C57BL/6 *gld* mice, suggesting that peripheral accumulation of these abnormal cells is retarded in mice lacking TNF. Importantly, our previous study showed that TNF also contributed to the mortality associated with *gld*, as only 4.1% of C57BL/6 *gld* TNF^{-/-} mice died by 420 days, compared with 31.9% of C57BL/6 *gld* mice.

Although TNF clearly contributes to the lymphoaccumulation and premature death associated with the *gld* phenotype, the mechanism used by TNF to exacerbate disease has not been determined. TNF is known to promote lymphocyte trafficking to, and within, lymphoid organs by inducing chemokine production (Ngo et al., 1999; Sedgwick et al., 2000), and in so doing might permit the homing and accumulation of B220⁺ DN T cells in *gld* mice. Alternatively, TNF has been shown to inhibit T cell apoptosis (Salmon et al., 1997; Vella et al., 1995) and might act antagonistically by inhibiting apoptosis of B220⁺ DN T cells, allowing an accumulation of these cells. C3H *gld* and BALB/c *gld* mice have been reported to develop plasmacytomas with age (Davidson et al., 1998), and TNF can stimulate the growth of B cell tumors *in vivo* by inducing IL-6 production (Carter et al., 1990). Here we show that TNF contributes to the premature death in *gld* mice by promoting plasmacytoma development.

Herein, we investigate TRAIL as a candidate molecule for regulation of the *gld* disease. TRAIL preferentially induces apoptosis in transformed, but not normal

cells (Nagata, 1997; Pitti et al., 1996; Wiley et al., 1995), although activated lymphocytes (Marsters et al., 1996; Ursini-Siegel et al., 2002; Wang et al., 2000) and APCs such as monocytes and DCs (Griffith et al., 1999; Hayakawa et al., 2004; Wang et al., 1999) may also be susceptible to TRAIL-mediated killing. Although lymphocyte homeostasis is normal in TRAIL-deficient mice, we hypothesized that TRAIL might contribute to the *gld* phenotype by inducing apoptosis in T or B lymphocytes and/or APCs to negatively regulate immune responses in *gld* mice. We report that in contrast to TNF, TRAIL has no effect on lymphoaccumulation, development of B220⁺ DN T cells, production of autoantibodies, or hypergammaglobulinemia associated with the *gld* phenotype.

4.3 Materials and Methods

Mice

Inbred C57BL/6 WT mice were purchased from The WEHI and C57BL/6 TRAIL^{-/-} mice were obtained and bred at Peter Mac as detailed in Chapter 2, Section 2.3, Materials and Methods. C57BL/6 *gld* (FasL mutant) (Cohen and Eisenberg, 1991; Davidson et al., 1986), C57BL/6 TNF-deficient (C57BL/6 TNF^{-/-}) mice (Korner et al., 1997) and C57BL/6 *gld* TNF^{-/-} mice (Korner et al., 2000) were obtained from Dr. Heinrich Korner, the Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia and bred at Peter Mac. C57BL/6 TRAIL^{+/+} *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were established by the author at Peter Mac by crossing C57BL/6 *gld* (n=10 backcrossed to C57BL/6) and C57BL/6 TRAIL^{-/-} mice (n=5 backcrossed to C57BL/6) and subsequently interbreeding the F₁ generation. This interbreeding enabled the generation of C57BL/6 TRAIL^{+/+} *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice on genetically very closely matched backgrounds. This was an important consideration, as the background strain of *gld* mice can affect severity of disease (Izui et al., 1984). Mice 4-28 weeks of age were used in all experiments, excluding aging experiments where mice were aged until moribund and then culled. Experiments were completed according to Animal Experimental Ethics Committee guidelines and approval and undertaken in specific pathogen-free conditions.

Organ weights/histology

Mice were weighed and then sacrificed at the ages indicated. Spleen, mLNs and axillary (a) LNs were excised and weighed wet. Organ weights of 6 mice (3 male, 3 female) were used for each time point. For aging experiments, mice were sacrificed and spleen and aLNs were weighed wet. A full autopsy was performed at sacrifice and routinely LNs, spleen and liver were examined by histology (hematoxylin and eosin (H&E) staining) after fixing these tissues in formalin. The preparation and staining of sections for histology and diagnosis of plasmacytoma development in aged mice were carried out by Dr. Duncan MacGregor (Department of Anatomical Pathology, Austin Hospital, Heidelberg,

Victoria, Australia). A total of 52 C57BL6 *gld* and 46 C57BL/6 *gld* TNF^{-/-} mice were aged.

Serum enzyme-linked immunosorbent assays (ELISAs)

Mice were culled at various time points. Six mice (3 male, 3 female) were used for each time point. Sera were tested by ELISA for total IgG, total anti-dsDNA antibodies and IgG1-specific anti-dsDNA antibodies. (a) IgG: ELISA plates (Costar, Cambridge, MA, USA) were pre-coated with 10 µg/ml goat anti-mouse IgG (Sigma Chemical Co.) in PBS for 1 h at RT. Plates were then washed 7 times in 0.05% Tween-20/PBS (TPBS) and blocked with 2% BSA/PBS for 1 h at room temperature. Subsequent steps with intervening washes included adding sera for 1 h at RT, horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse Ig (multiply adsorbed) (BD Biosciences Pharmingen) for 1 h at RT and development with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) for 10-20 min. (read at OD 405 nm). (b) Total anti-dsDNA: Maxi-Sorp ELISA plates (Nalge Nunc International, Roskilde, Denmark) were pre-coated with 10 µg/ml methylated BSA (Sigma Chemical Co.) in 0.2 M carbonate buffer overnight at 4°C. After 4 washes in TPBS, 10 µg/ml herring sperm DNA was added in carbonate buffer overnight at 4°C. Plates were washed and blocked with 2% polyethylene glycol 8,000/1% gelatine/1% BSA in TPBS for 30 min. at 37°C. Subsequent steps with intervening washes included adding sera for 2 h at 37°C, HRP-conjugated polyclonal goat anti-mouse Ig (multiply adsorbed) for 1 h at 37°C and development with ABTS for 10-20 min. (read at OD 405 nm). (c) IgG1 anti-dsDNA: ELISAs were performed the same as for total anti-dsDNA, except HRP-conjugated rat anti-mouse IgG1 (Zymed Laboratories, South San Francisco, CA, USA) was used in place of HRP-conjugated goat anti-mouse Ig (multiply adsorbed). The mean level of serum anti-dsDNA antibodies of 28 week C57BL/6 TRAIL^{+/+} *gld* mice was set at 100% and the results of other genotypes were recorded in relation to this level.

Flow cytometric analysis

The following mAb were used in multi-parameter flow cytometric analysis: anti-mTRAIL-PE (N2B2), rat IgG2a isotype control-PE, anti-mouse DR5-biotin

(MD5-1), anti-mouse CD3-PE or -Cy-Chrome (17A2), anti-mouse TCR β -FITC (H57-597), anti-mouse B220-APC (RA3-6B2), anti-mouse CD4-PE or -FITC (RM-4.5), anti-mouse CD8 α -PE-Cy5.5, -PE or -FITC (53-6.7) followed by incubation with streptavidin-PE or streptavidin-APC-Cy7. To avoid the non-specific binding of mAbs to Fc γ R, anti-mouse CD16/32 (2.4G2) mAb was added to the mAb cocktail. Anti-mTRAIL-PE was obtained from e-Bioscience, anti-DR5-biotin was kindly provided by Dr. Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan), anti-CD8 α -PE-Cy5.5 was sourced from Caltag and the remaining reagents from BD Biosciences Pharmingen. After washing the cells with PBS/FCS/azide, cells were analyzed on a FACScanTM or FACSDiva and the data processed by either CELLQuestTM or FCS-Express 2 software.

Statistical analysis

Data were analyzed using a two tailed, non-parametric, unpaired Mann Whitney test. P values < 0.05 were considered significant.

4.4 Results

4.4.1 Expression of TRAIL and TRAIL receptor (DR5) in the spleen of *gld* mice.

To explore the possibility that TRAIL might contribute to the *gld* phenotype by inducing apoptosis in T or B lymphocytes, expression of TRAIL and TRAIL receptor (DR5) was examined on various cell types within the spleen of C57BL/6 *gld* mice. If TRAIL-mediated apoptosis is important for lymphocyte homeostasis in *gld* mice, DR5 expression might be expected on target lymphocyte populations and TRAIL expression might be predicted on effector cell populations within the spleen. However, TRAIL and DR5 expression were not observed on B220⁺ DN T cells, T cells, B cells or on B220⁺TCR β ⁺ cells in the spleen (Figure 4.3).

4.4.2 TRAIL has no effect on the lymphoaccumulation associated with *gld*

To evaluate whether TRAIL contributes to the lymphoaccumulation associated with the *gld* phenotype, spleen, aLN and mLN weight were compared in C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. Spleen and mLN weight gradually increased in C57BL/6 *gld* mice from 4 weeks of age, whereas aLN weight increased from 12 weeks of age (Figure 4.4). Lymphoaccumulation was equivalent in C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice (Figure 4.4), whereas C57BL/6 mice did not show obvious signs of lymphoproliferation, as expected (Figure 4.4). Total body weight increased over the period of observation, but was equivalent in all strains tested (Figure 4.4). Together, these data suggest that unlike TNF, TRAIL has no effect on the generalized lymphoproliferation associated with the *gld* phenotype.

4.4.3 Accumulation of B220⁺TCR $\alpha\beta$ ⁺CD4⁻CD8⁻ double negative (DN) T cells in TRAIL^{-/-} *gld* spleen and peripheral blood lymphocytes (PBL)

Lymphoproliferative disease in *gld* mice is characterized by an accumulation of B220⁺ DN T cells in LNs, spleen and PBL (Cohen and Eisenberg, 1991) that are typically eliminated by Fas/FasL interactions. To investigate whether TRAIL plays a role in the accumulation or localization of these cells in *gld* mice, flow cytometric analysis was performed to compare proportions of these cells in the

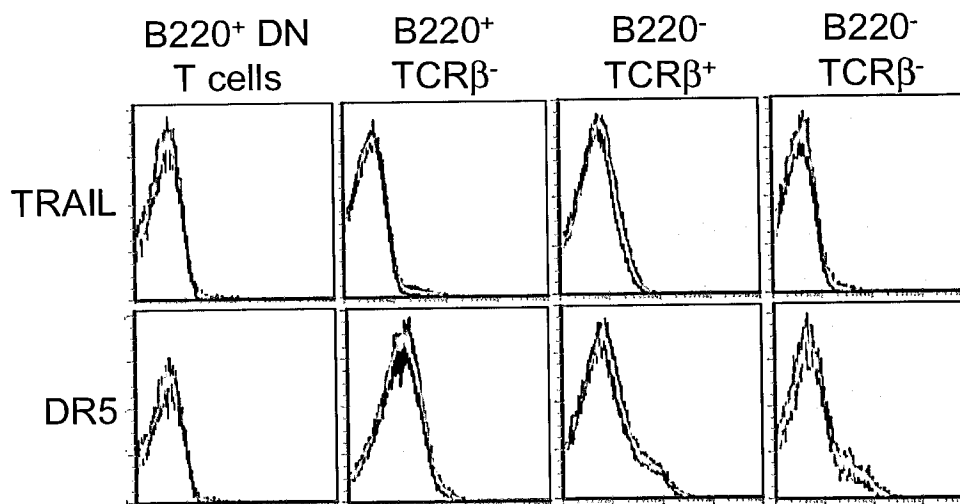


Figure 4.3. Lack of TRAIL and TRAIL receptor (DR5) expression in the spleen of *gld* mice. Splenocytes isolated from adult (> 8 weeks) C57BL/6 *gld* mice were analyzed by flow cytometry for TRAIL and TRAIL receptor (DR5) expression. Black histograms represent isotype control staining and red histograms represent TRAIL or DR5 staining as indicated. Results shown are representative of two independent experiments, each containing one C57BL/6 *gld* mouse.

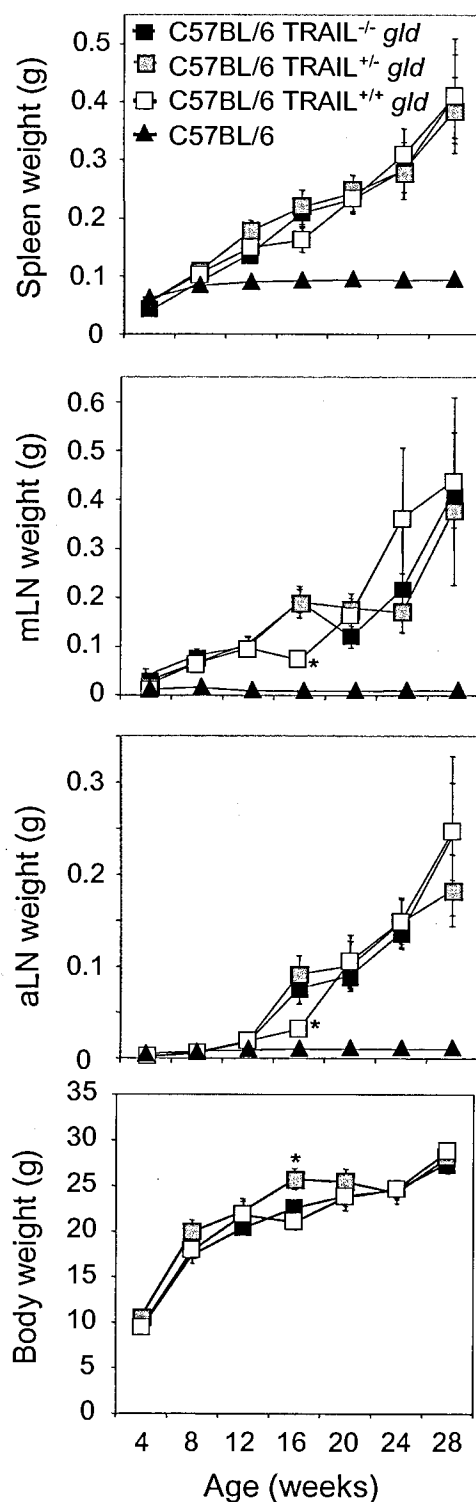


Figure 4.4. Lymphoid organ and body weights for C57BL/6, C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. Spleen, aLN, mLN and total body weight are shown in grams (g) for C57BL/6, C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice at 4, 8, 12, 16, 20, 24 and 28 weeks of age. Data represent mean \pm SEM of six mice (3 female, 3 male) at each time point, for each genotype. Asterisks indicate groups of *gld* mice that are significantly different (* P < 0.02).

blood and spleen of C57BL/6 WT, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. At 5 weeks of age, proportions of B220⁺ DN T cells were negligible in the blood (Figure 4.5 and 4.7A) and spleen (Figures 4.6 and 4.7B), irrespective of the mouse genotype. By 12 weeks of age, proportions of B220⁺ DN T cells in the blood (Figure 4.5 and 4.7A) and spleen (Figures 4.6 and 4.7B) increased to ~15% in C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. By 20 weeks of age, proportions of B220⁺ DN T cells increased to ~35% in the blood (Figure 4.5 and 4.7A) and ~25% in the spleen (Figure 4.6 and 4.7B) of C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. B220⁺ DN T cells did not accumulate in C57BL/6 WT or C57BL/6 TRAIL^{-/-} mice. Comparable proportions of B220⁺ DN T cells in the blood and spleen of TRAIL sufficient and TRAIL deficient *gld* mice suggests a negligible role for TRAIL in the accumulation and localization of these abnormal lymphocytes.

4.4.4 Proportions of T and B cells in TRAIL^{-/-} *gld* spleen and PBL

We then examined whether accumulation of B220⁺ DN T cells in C57BL/6 *gld* or C57BL/6 TRAIL^{-/-} *gld* mice had an effect on proportions of classical T or B cells (identified as TCRβ⁺ and B220⁺ cells but excluding abnormal B220⁺ DN T cells) in the spleen and blood of these animals. Proportions of T cells in the spleen of 5, 12, and 20 week old C57BL/6 WT, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were equivalent and ranged from ~20-30% (Table 4.1). In contrast, proportions of B cells were decreased in the spleen of C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice from 12 weeks of age (Table 4.1), which coincided with an accumulation of B220⁺ DN T cells in the spleen. In the blood, similar proportions of T and B cells were observed in 5, 12, and 20 week old C57BL/6 WT, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice, although 5 week old C57BL/6 TRAIL^{-/-} *gld* mice appeared to have elevated proportions of T cells and decreased proportions of B cells compared to the other genotypes. Further experiments, using greater numbers of mice are required to determine whether this finding is reproducible.

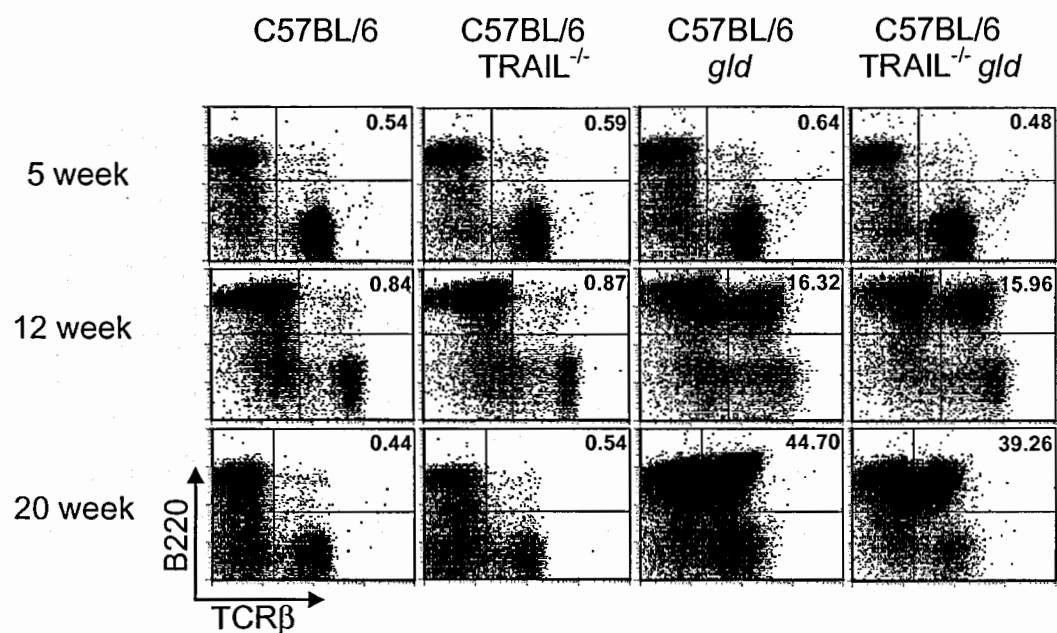


Figure 4.5. TCR β versus B220 expression on PBLs. C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were sacrificed at 5, 12 and 20 weeks of age, PBLs isolated and stained with antibodies to B220 and TCR β . Numbers represent percentage B220⁺TCR β ⁺ cells for each profile. Data are representative from the analysis of 3 C57BL/6 *gld*, 3 C57BL/6 TRAIL^{-/-} *gld*, 3 C57BL/6 and 3 C57BL/6 TRAIL^{-/-} mice for each age group, except at 5 weeks where 1 C57BL/6 and 1 C57BL/6 TRAIL^{-/-} mouse were analyzed.

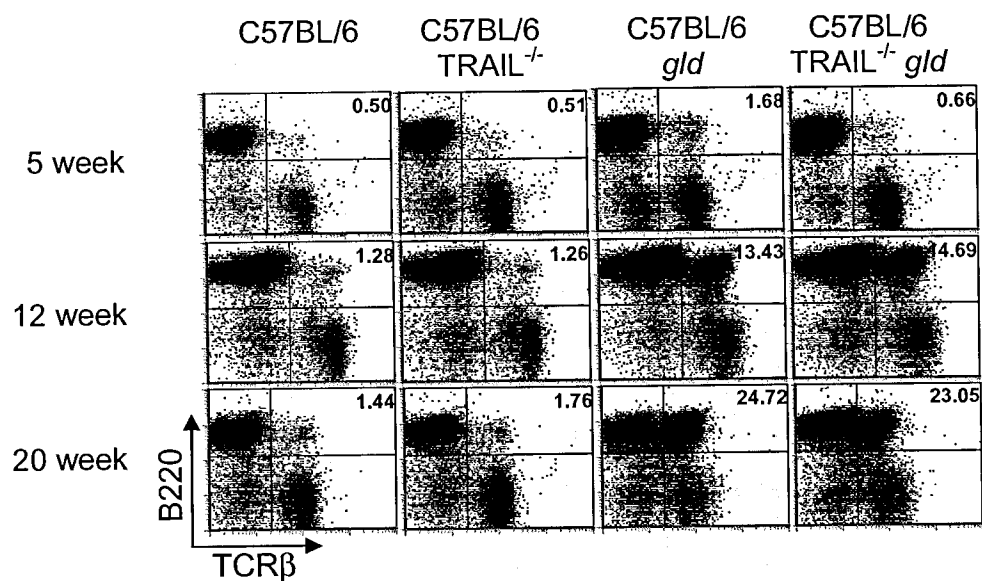


Figure 4.6. TCR β versus B220 expression on splenocytes. C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were sacrificed at 5, 12 and 20 weeks of age, their splenocytes isolated and stained with antibodies to B220 and TCR β . Data are representative from the analysis of 3 C57BL/6 *gld*, 3 C57BL/6 TRAIL^{-/-} *gld*, 3 C57BL/6 and 3 C57BL/6 TRAIL^{-/-} mice for each age group, except at 5 weeks where 1 C57BL/6 and 1 C57BL/6 TRAIL^{-/-} mouse were analyzed.

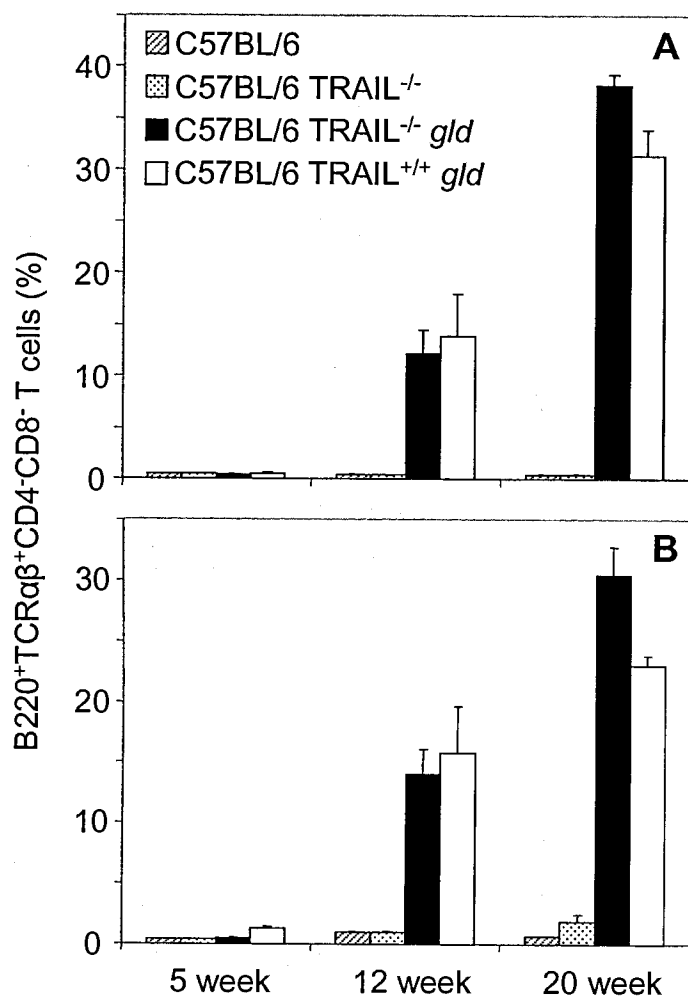


Figure 4.7. Age-related accumulation of B220⁺TCRαβ⁺CD4⁺CD8⁻ DN T cells in PBL and spleen. C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were sacrificed at 5, 12 and 20 weeks of age, their splenocytes and PBLs isolated and stained with antibodies to B220, TCRβ, CD4 and CD8. Percentage B220⁺TCRαβ⁺CD4⁺CD8⁻ T cells is shown for PBL (A) and spleen (B). Data represent mean ± SEM for 3 C57BL/6 *gld*, 3 C57BL/6 TRAIL^{-/-} *gld*, 3 C57BL/6 and 3 C57BL/6 TRAIL^{-/-} mice for each age group, except at 5 weeks when 1 C57BL/6 and 1 C57BL/6 TRAIL^{-/-} mouse were analyzed. Proportions of B220⁺TCRαβ⁺CD4⁺CD8⁻ DN T cells in C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* spleen or PBL are not significantly different ($P > 0.1$) at any of the time points shown.

PBL

	5 weeks		12 weeks		20 weeks	
	T cells (%)	B cells (%)	T cells (%)	B cells (%)	T cells (%)	B cells (%)
C57BL/6	45.28	37.82	27.71 \pm 1.46	59.18 \pm 1.93	27.46 \pm 2.20	46.27 \pm 6.40
C57BL/6 TRAIL ^{-/-}	51.84	38.27	24.46 \pm 0.64	61.53 \pm 0.93	35.51 \pm 6.96	28.79 \pm 2.87
C57BL/6 <i>gld</i>	44.88 \pm 3.45	34.54 \pm 1.83	33.37 \pm 2.68	40.88 \pm 0.44	21.85 \pm 2.71	37.53 \pm 5.28
C57BL/6 TRAIL ^{-/-} <i>gld</i>	63.77 \pm 1.64	17.61 \pm 3.42	36.62 \pm 1.13	42.17 \pm 2.15	23.38 \pm 2.93	36.86 \pm 4.29

Spleen

	5 weeks		12 weeks		20 weeks	
	T cells (%)	B cells (%)	T cells (%)	B cells (%)	T cells (%)	B cells (%)
C57BL/6	20.43	70.66	22.86 \pm 0.76	65.45 \pm 2.40	28.05 \pm 2.67	56.52 \pm 3.79
C57BL/6 TRAIL ^{-/-}	20.42	66.78	23.35 \pm 0.82	67.25 \pm 0.60	27.75 \pm 3.66	51.81 \pm 3.89
C57BL/6 <i>gld</i>	21.46 \pm 0.45	64.19 \pm 2.20	21.76 \pm 2.20	53.19 \pm 2.74	22.41 \pm 2.10	44.22 \pm 2.19
C57BL/6 TRAIL ^{-/-} <i>gld</i>	25.17 \pm 1.03	60.84 \pm 4.51	24.90 \pm 0.71	51.35 \pm 1.89	20.32 \pm 1.42	35.77 \pm 5.26

Table 4.1. Proportional changes in T and B cells in the blood and spleen. PBL and spleen were isolated from C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice and stained with antibodies to B220, TCR β , CD4 and CD8. Percentage T and B cells was calculated by subtracting the percentage of B220⁺TCR $\alpha\beta$ ⁺CD4⁻CD8⁻ DN T cells present from the percentage of total TCR β ⁺ and total B220⁺ cells. Data represent mean \pm SEM for 3 C57BL/6 *gld*, 3 C57BL/6 TRAIL^{-/-} *gld*, 3 C57BL/6 and 3 C57BL/6 TRAIL^{-/-} mice for each age group, except at 5 weeks when only 1 C57BL/6 and 1 C57BL/6 TRAIL^{-/-} mouse were analyzed.

4.4.5 Levels of serum IgG and anti-double stranded (ds) DNA Abs in TRAIL^{-/-} *gld* mice

Hypergammaglobulinemia and circulating autoantibodies are features of the *gld* phenotype (Cohen and Eisenberg, 1991). Given that TRAIL has previously been shown to induce apoptosis in B lymphocytes (Ursini-Siegel et al., 2002; Wang et al., 2000), we were interested in the effect that TRAIL might have on antibody production in *gld* mice. Concentration of serum IgG and anti-ds DNA autoantibody were compared in C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice. The concentration of IgG in the serum increased dramatically until 12-16 weeks of age, regardless of the genotype (Figure 4.8A). Serum anti-dsDNA autoantibody levels also increased with age, regardless of the genotype (Figure 4.8B). Interestingly, although serum IgG concentrations peaked after 12-16 weeks of age, anti-dsDNA antibody levels continued to increase, even at 28 weeks of age (Figure 4.8B). Given that serum autoantibody levels of the IgG1 subclass were reported to be elevated in *gld* mice administered with a neutralizing anti-mTRAIL mAb (Kayagaki et al., 2002), we examined the effect of TRAIL deficiency on anti-dsDNA IgG1 autoantibody levels. Serum IgG1 anti-dsDNA antibody levels increased with age, regardless of phenotype (Figure 4.8C). However, considerable variability in IgG1 autoantibody production was observed between mice, suggesting that larger group sizes may need to be analyzed to obtain statistically significant differences between genotypes. Analysis of circulating autoantibodies with different specificities may also be required to ascertain whether IgG1 subclass autoantibodies are elevated in C57BL/6 TRAIL^{-/-} *gld* mice.

4.4.5 Phenotype of aged TNF^{-/-} *gld* and TNF^{+/+} *gld* mice

Previous studies undertaken in our laboratory have identified a critical contribution for TNF in the lymphoaccumulation and mortality associated with the *gld* phenotype (Korner et al., 2000). To investigate the mechanism used by TNF to promote premature death, C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice were aged and lymphoaccumulation was compared in these mice (Appendix III and IV). C57BL/6 *gld* TNF^{-/-} mice had an increased life expectancy (63.9 ± 2.4 weeks, n=46) compared with C57BL/6 *gld* mice (48.4 ± 1.6 weeks, n=52)

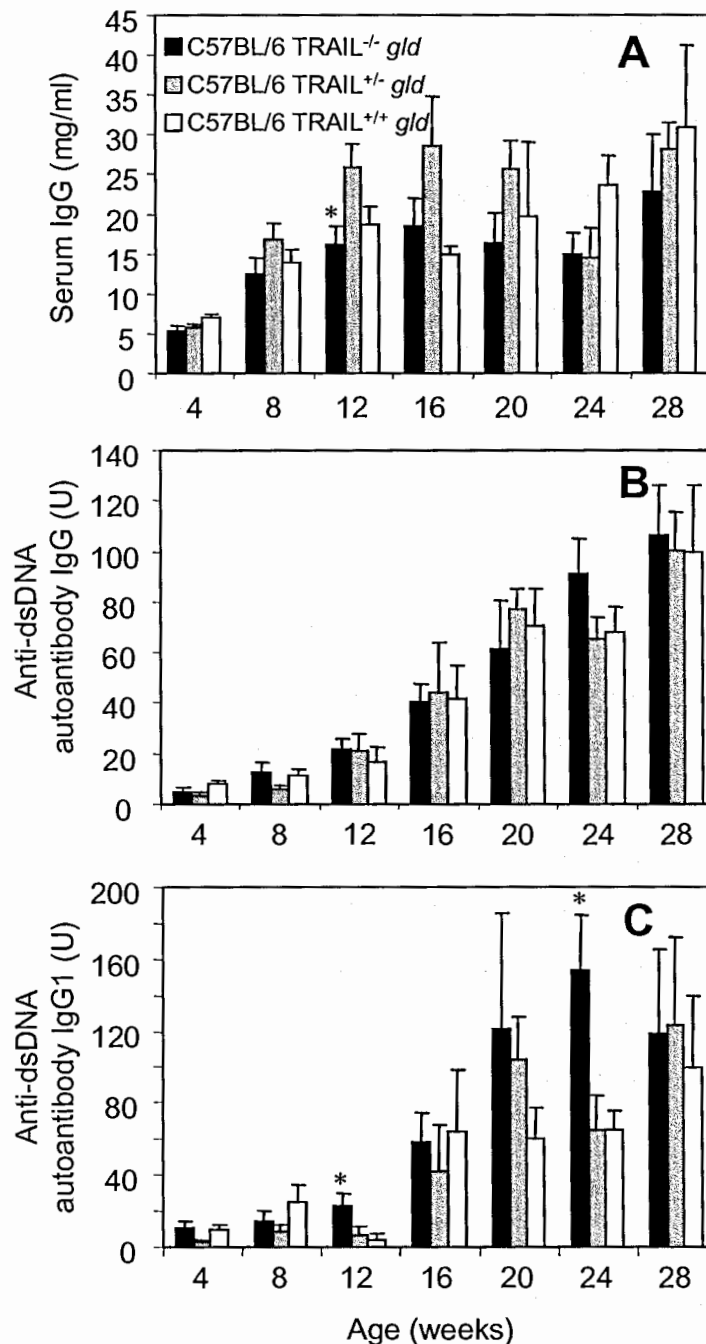


Figure 4.8. Effect of TRAIL deficiency on serum IgG and autoantibody concentration in *gld* mice. Serum was isolated from C57BL/6 *gld*, C57BL/6 TRAIL^{+/-} *gld* and C57BL/6 TRAIL^{-/-} *gld* mice at 4, 8, 12, 16, 20, 24 and 28 weeks of age and tested by ELISA for (A) total concentration of IgG, (B) total anti-dsDNA autoantibodies and (C) IgG1 anti-dsDNA autoantibodies. The concentration of anti-dsDNA autoantibodies are shown relative to that observed for 28 week C57BL/6 *gld* mice (100 Units was defined as the mean of absorbance at 405nm for 28 week C57BL/6 *gld* mice). Mean \pm SEM were calculated from the analysis of six mice (3 female, 3 male) at each time point, for each genotype. Asterisks indicate groups of C57BL/6 TRAIL^{-/-} *gld* mice that are significantly different to C57BL/6 *gld* or C57BL/6 TRAIL^{+/-} *gld* mice of the same age (* $P < 0.05$).

($P < 0.01$, Figure 4.9 and 4.10). Moreover, secondary lymphoid organ (aLN and spleen) weight was significantly reduced in C57BL/6 *gld* $TNF^{-/-}$ compared with C57BL/6 *gld* mice at death ($P < 0.01$, Figure 4.10), although total body weights were equivalent. This suggested that the cause of death, at least in C57BL/6 *gld* $TNF^{-/-}$ mice, was not a direct consequence of lymphoaccumulation.

4.4.6 TNF is not required for plasmacytoma development in aged *gld* mice

C3H *gld* and BALB/c *gld* mice have been reported to develop plasmacytomas with age (Davidson et al., 1998), and TNF is known to stimulate the growth of some B cell tumors *in vivo* by inducing IL-6 production (Carter et al., 1990). To investigate whether TNF contributes to premature death in *gld* mice by promoting plasmacytoma development, LN, spleen and liver sections from C57BL/6 *gld* and C57BL/6 *gld* $TNF^{-/-}$ mice were examined by histology (H&E staining) after fixing these tissues in formalin. Histological analysis revealed the presence of malignant lymphoma in all C57BL/6 *gld* and C57BL/6 *gld* $TNF^{-/-}$ mice evaluated at death. Moreover, lymphoid cells with plasmacytoid nuclear (medium to large size nuclei, coarse chromatin and irregular nuclear outlines) and cytoplasmic features were identified in these mice (Figure 4.11). Plasmacytoma was diagnosed in a minimum of one organ per mouse, and often in multiple organs of C57BL/6 *gld* and C57BL/6 *gld* $TNF^{-/-}$ mice diagnosed with malignant lymphoma. Plasmacytoma was identified in a variety of LNs, spleen and the liver. Representative H&E stained spleen, liver and LN are shown from a single C57BL/6 *gld* and C57BL/6 *gld* $TNF^{-/-}$ mouse, illustrating that plasmacytoid features often presented in more than one organ per mouse (Figure 4.12). Together these data suggest that *gld* mice deficient in TNF are capable of developing plasmacytomas.

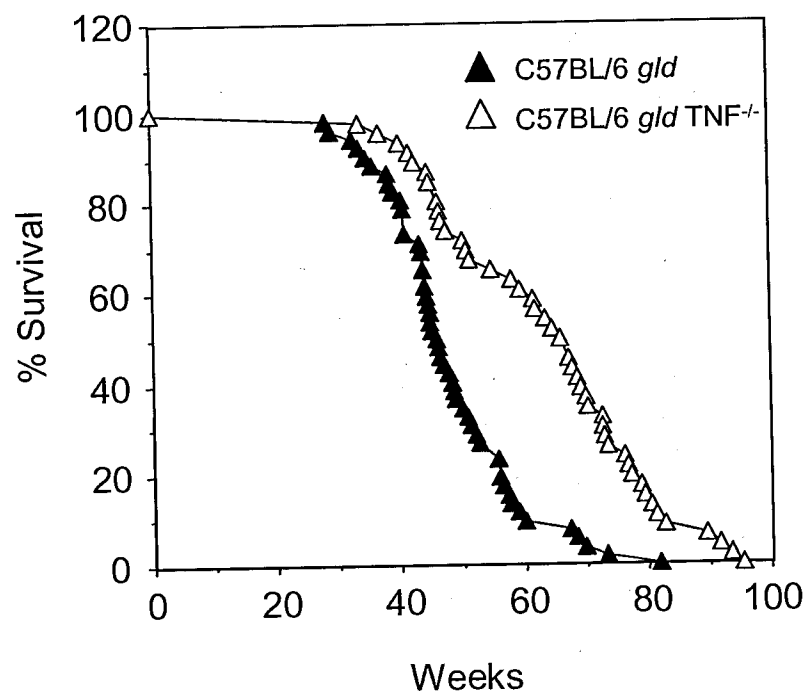


Figure 4.9. Survival plot for aged C57BL/6 *gld* and C57BL/6 *gld* $TNF^{-/-}$ mice. Mean lifespan \pm SEM was 48.5 ± 1.6 weeks for C57BL/6 *gld* mice ($n=52$) and 63.9 ± 2.4 weeks for C57BL/6 *gld* $TNF^{-/-}$ mice ($n=46$).

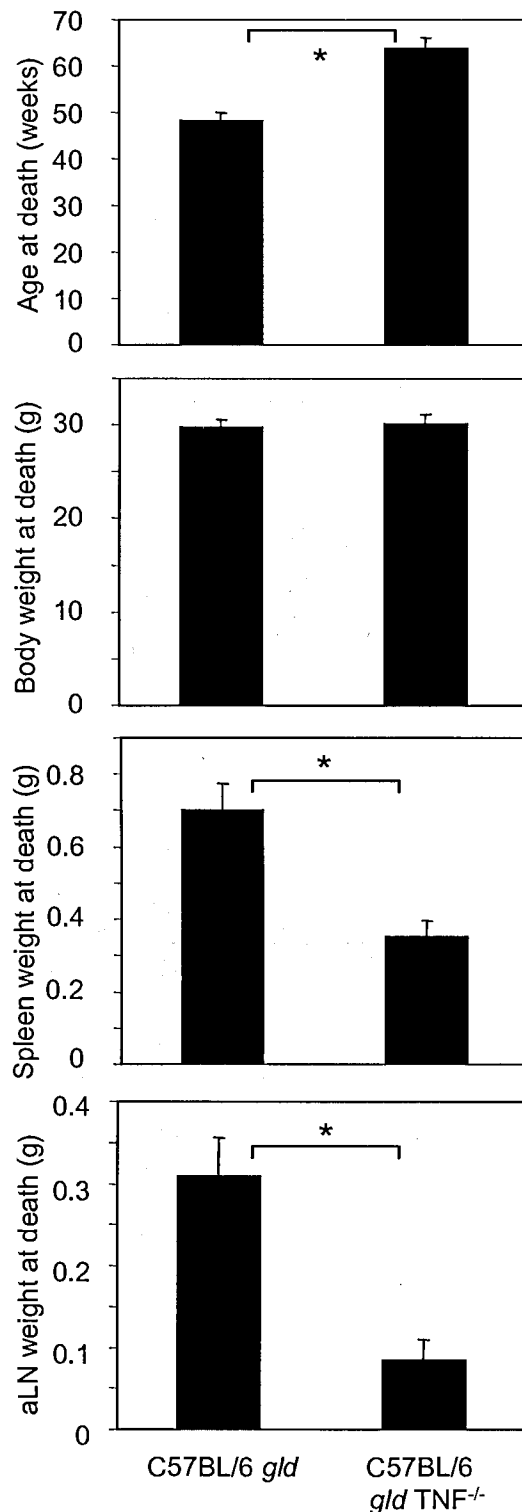


Figure 4.10. Increased life expectancy and decreased lymphoaccumulation in aged C57BL/6 *gld* $TNF^{-/-}$ mice. C57BL/6 *gld* mice and C57BL/6 *gld* $TNF^{-/-}$ mice were aged until death and a full autopsy performed. (A) Age, (B) body weight, (C) spleen weight and (D) mean aLN weight at death are shown. Mean \pm SEM were calculated from analysis of 52 C57BL/6 *gld* and 46 C57BL/6 *gld* $TNF^{-/-}$ mice (for age at death), and from analysis of 38 C57BL/6 *gld* and 27 C57BL/6 *gld* $TNF^{-/-}$ mice (for body and organ weights). Asterisks indicate groups that are significantly different (* $P < 0.01$).

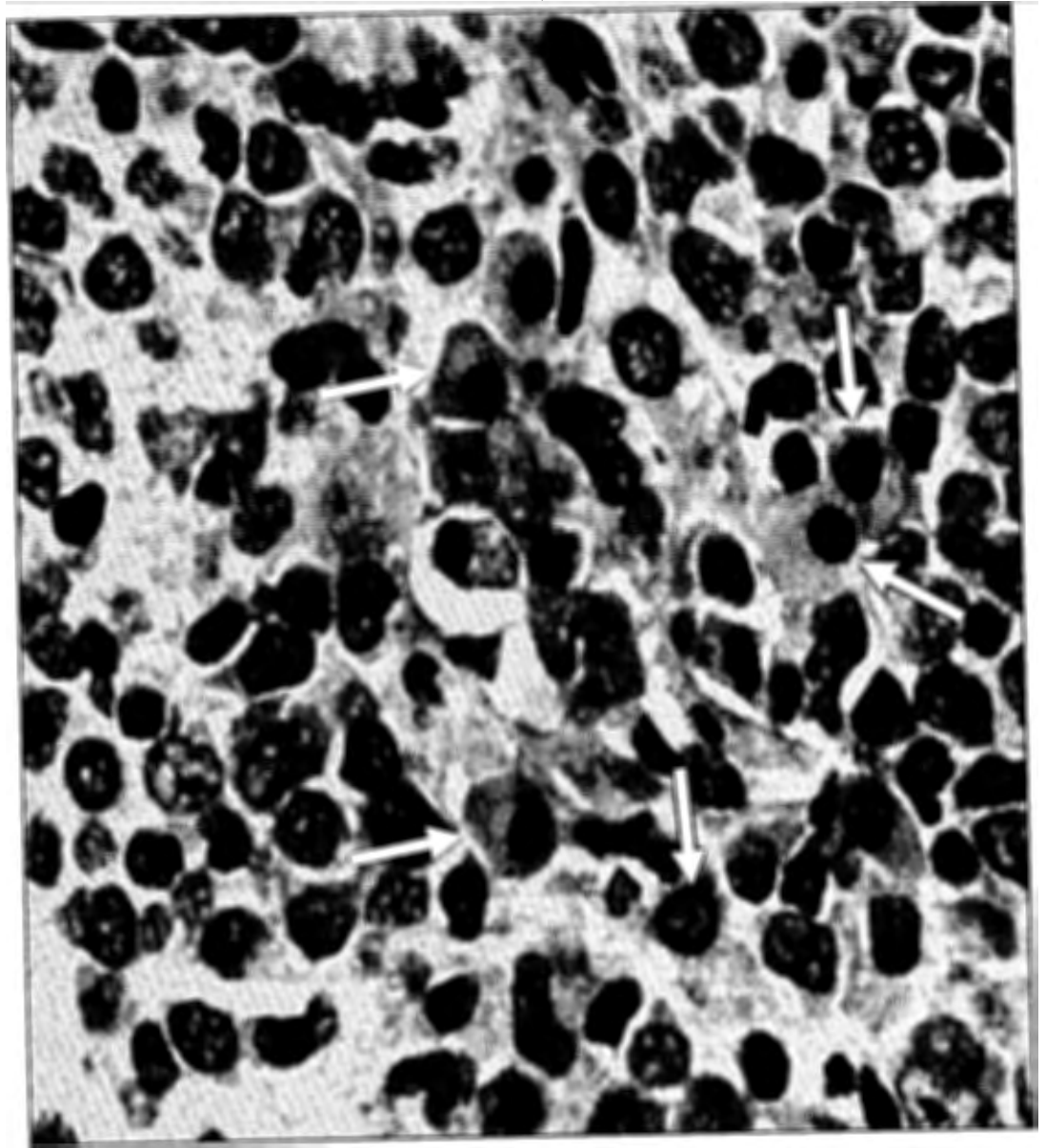


Figure 4.11. Comparison of the phenotypic characteristics of mature plasma cells and atypical plasmacytoid cells. Representative H&E staining on mLN isolated from an aged C57BL/6 *gld* mouse. Yellow arrows indicate mature plasma cells, that are characterized by a prominent nucleolus, peripheral chromatin clumping and a paranuclear clearing (or "hof"), as well as eccentric cytoplasm. White arrows indicate atypical plasma cells (plasmacytoid cells) characterized by medium to large size nuclei, coarse chromatin and irregular nuclear outlines. (Original magnification x 40).

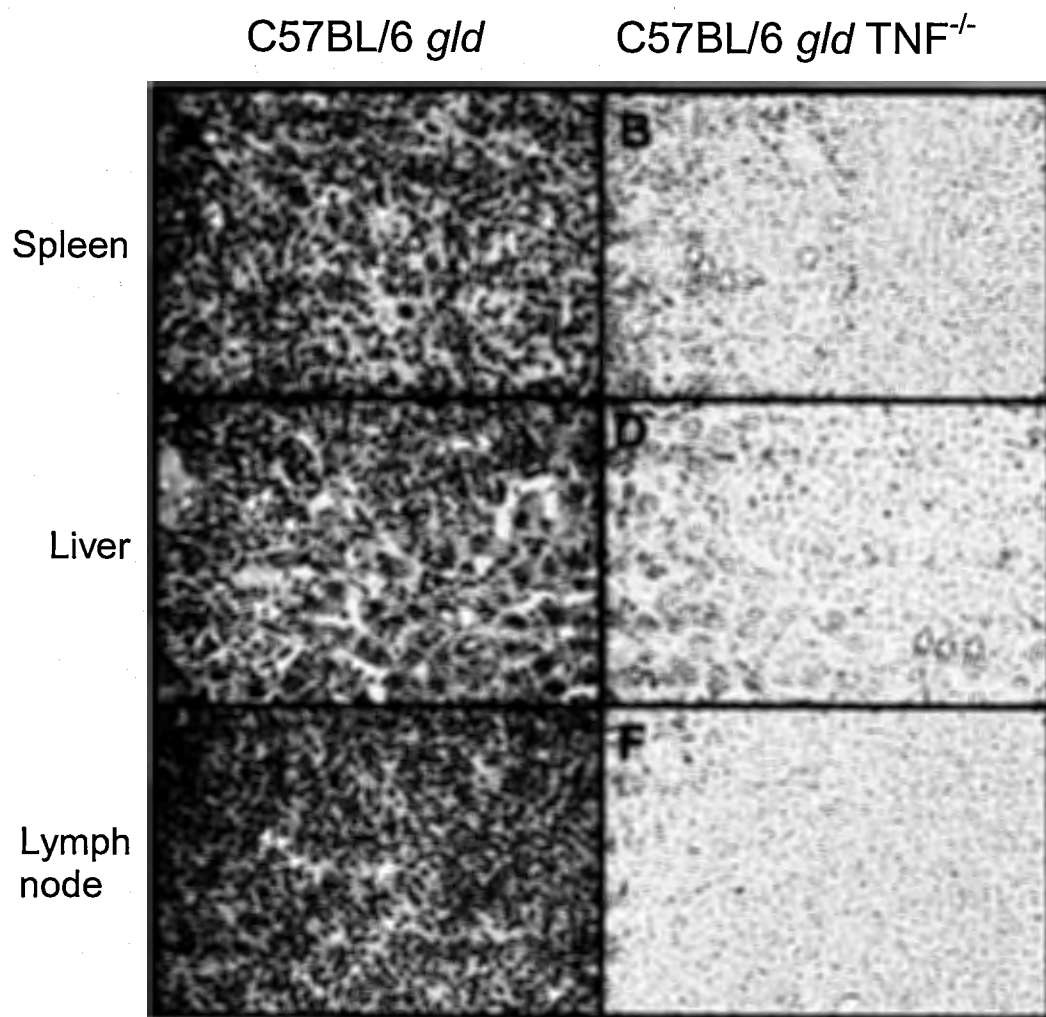


Figure 4.12. Malignant lymphoid infiltrates in the tissues of aged C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice. Representative H&E staining on spleen, liver and LN isolated from aged C57BL/6 *gld* (A, C, E) and C57BL/6 *gld* TNF^{-/-} (B, D, F) mice at death. Histological analysis reveals the presence of malignant lymphoma in spleen, liver and LN of C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice. The presence of lymphoid cells with plasmacytoid nuclei and cytoplasmic features suggests a diagnosis of plasmacytoma in these mice. Yellow arrows indicate mature plasma cells, whereas white arrows indicate atypical plasma cells (plasmacytoid cells). Histology depicted is of organs isolated from one C57BL/6 *gld* and one C57BL/6 *gld* TNF^{-/-} mouse and are representative from the analysis of 5 aged C57BL/6 *gld* and 5 aged C57BL/6 *gld* TNF^{-/-} mice. Original magnification x 10.

4.5 Discussion

TNF was the first specific molecule reported to contribute to the lymphoaccumulation and premature death associated with the *gld* phenotype (Korner et al., 2000). This finding has important therapeutic applications, as strategies to block TNF function may suppress lymphoaccumulation and increase the life expectancy of patients with lymphoproliferative disorders such as ALPS. The chemokine monocyte chemoattractant protein (MCP)-1 has been reported to contribute to some features of the *lpr* phenotype, such as autoimmune disease and lymphadenopathy, but not others such as splenomegaly or hypergammaglobulinemia (Tesch et al., 1999). TNF is known to induce the production of chemokines (Ngo et al., 1999; Sedgwick et al., 2000) and may act upstream of MCP-1 or other chemokines to regulate disease in *gld* or *lpr* mice.

In the present study, the potential of TRAIL to regulate the *gld* phenotype was investigated. When TRAIL was deleted in C57BL/6 *gld* mice by gene-targeting, lymphoaccumulation in secondary lymphoid organs, and accumulation of B220⁺ DN T cells in the blood and spleen were unaffected. Moreover, hypergammaglobulinemia and circulating autoantibodies were unchanged in these mice. Thus, although TRAIL has been reported to induce apoptosis in activated B and T lymphocytes (Marsters et al., 1996; Ursini-Siegel et al., 2002; Wang et al., 2000), surprisingly TRAIL does not appear to play a major role in regulation of the *gld* disease.

During the course of our study, it was reported by Kayagaki and colleagues that administration of neutralizing anti-mTRAIL mAb had no effect on lymphoaccumulation or the development of B220⁺ DN T cells in C3H/HeJ *gld* mice (Kayagaki et al., 2002). Whilst administration of neutralizing anti-mTRAIL mAb is an effective means of blocking TRAIL activity *in vivo*, TRAIL neutralization in the Kayagaki et al. study was only commenced at 3 weeks of age. If TRAIL influences the *gld* phenotype prior to 3 weeks of age, the full extent of the contribution by TRAIL to the *gld* phenotype would not be recognized in the Kayagaki et al. study. Nonetheless, our characterization of

C57BL/6 TRAIL^{-/-} *gld* mice also failed to reveal a contribution by TRAIL to the *gld* phenotype. In contrast to our findings, TRAIL neutralization was shown to increase serum autoantibody levels of the IgG1 subclass in C3H/HeJ *gld* mice (Kayagaki et al., 2002). This difference may be due to the comparative sensitivity of C57BL/6 *gld* and C3H/HeJ *gld* mice to development of autoimmune disease, although this is yet to be tested. Interestingly, antibody production was reported to be decreased in mice immunized with TRAIL expressing antigen-bearing APCs, suggesting that APCs can suppress antibody production *in vivo* in a TRAIL-dependent manner (Kayagaki et al., 2002).

The severity of the *gld* phenotype is greater in highly susceptible C3H/HeJ *gld* mice (Izui et al., 1984), compared to less susceptible C57BL/6 *gld* mice. Whilst highly susceptible C3H/HeJ *gld* mice were analyzed by Kayagaki et al., our study demonstrated that TRAIL does not contribute to the lymphoaccumulation associated with a less severe form of *gld* disease in C57BL/6 *gld* mice. Examining disease development in both highly permissive and less permissive strains of *gld* mice is critical to gain a full understanding of the mechanisms regulating disease development. Considerable variation in severity of disease is evident between individual ALPS patients and between ALPS patients of different classifications (ALPS 0, ALPS Ia, ALPS Ib, ALPS II and ALPS III) (Rieux-Laucat et al., 2003). Accordingly, the molecular mechanisms involved in regulation of the disease might vary depending on the severity of symptoms in these patients.

In Chapter 5, we use TRAIL-deficient mice and a neutralizing anti-mTRAIL mAb to identify a role for TRAIL in suppression of EAE. In that model, we demonstrate that TRAIL suppressed T cell proliferation, but had no effect on antibody production associated with disease. Other researchers have used soluble TRAIL receptor (DR5) to block TRAIL function and to similarly identify a role for TRAIL in suppression of EAE. However, in that study, TRAIL had a negligible effect on lymphocyte proliferation (Hilliard et al., 2001). TRAIL has also been identified to suppress collagen-induced arthritis (Song et al., 2000) as well as cyclophosphamide and streptozotocin-induced diabetes (Lamhamedi-

Cherradi et al., 2003). In those experimental models, TRAIL was proposed to suppress both lymphocyte proliferation, and antibody production associated with disease (Lamhamedi-Cherradi et al., 2003; Song et al., 2000). In contrast, we observed no significant effect of TRAIL on lymphocyte proliferation or antibody production associated with the *gld* phenotype, suggesting that the role of TRAIL in disease is dependent on the pathological condition.

Although a previous study in our laboratory revealed that TNF contributes to the premature death associated with the *gld* disease, the study was limited as mice were only aged for a period of 420 days (Korner et al., 2000). In the current study, C57BL/6 *gld* and C57BL/6 *gld* TNF^{-/-} mice were aged until moribund (up to 667 days) to observe the full manifestation of lymphoproliferative disease in these mice. As expected, TNF-deficiency increased the lifespan of C57BL/6 *gld* mice. Moreover, lymphoaccumulation was decreased in C57BL/6 *gld* TNF^{-/-} compared to C57BL/6 *gld* mice at death. A number of treatments have been reported to decrease lymphoproliferative disease and accumulation of B220⁺ DN T cells in *lpr* mice, including chronic treatment with mAb specific for B220, CD4, CD8 or TCR V β , chronic administration of SEB or cyclosporine A and neonatal thymectomy (Asensi et al., 1989; de Alboran et al., 1992; Mountz et al., 1987; Santoro et al., 1988; Steinberg et al., 1980). Our findings suggest that TNF blockade or suppression of downstream chemotactic pathways might decrease the lymphoaccumulation and increase the life expectancy of ALPS patients.

A number of studies have implicated the Fas/FasL pathway in control of B cell tumor growth. Growth of Fas-sensitive A20 B cell tumor cells is accelerated when these cells are transfected with FLIP, suggesting that Fas can control malignant B cell tumor growth *in vivo* (Djerbi et al., 1999). In addition, tumor cells isolated from a number of B cell malignancies are resistant to Fas-induced death even though they express adequate cell surface Fas receptor (Owen-Schaub et al., 1993; Plumas et al., 1998; Westendorf et al., 1995; Xerri et al., 1998). In contrast, TNF is thought to contribute to the development of the B-cell malignancy multiple myeloma, by inducing IL-6 which acts as a major growth factor for multiple myeloma cells *in vitro* and *in vivo* (Carter et al., 1990).

Previous studies have shown that *gld* mice develop plasmacytomas with age (Davidson et al., 1998) and ALPS patients with Fas/FasL mutations are frequently diagnosed with non-Hodgkin's and Hodgkin's B cell malignancies (Lin, 1995). In the present study, we demonstrated that although C57BL/6 *gld* TNF^{-/-} mice have an increased life expectancy compared to C57BL/6 *gld* mice, both strains develop plasmacytomas. This suggests that TNF is not absolutely critical for the development of plasmacytomas in *gld* mice, but might promote plasmacytoma development, causing premature death. Histological analysis of tissue sections from C57BL/6 *gld* TNF^{-/-} and C57BL/6 *gld* mice at various time points prior to death will allow assessment of this hypothesis. In addition to plasmacytoma development, premature death in a proportion of *gld* mice might be direct consequence of the effects of lymphoaccumulation on the functioning of essential organs.

Although TRAIL can suppress tumor initiation, growth and metastasis, we were unable to identify a major role for TRAIL in regulation of the *gld* phenotype. We also revealed that TNF is not critical for plasmacytoma development in *gld* mice, but may promote the development of these tumors. The mechanism used by TNF to sustain the *gld* phenotype is yet to be discovered.

CHAPTER FIVE

TRAIL SUPPRESSES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

5.1 Abstract

TRAIL can selectively induce apoptosis in transformed and virus-infected cells, and suppress tumor initiation, growth and metastasis *in vivo*. In contrast, the role of TRAIL in non-transformed tissues is largely unknown. In Chapter 4, we failed to identify a role for TRAIL in regulation of the *gld* phenotype, but here we investigate TRAIL function in the regulation of autoimmune disease. A previous study suggested that TRAIL may suppress collagen-induced arthritis in mice. Herein, we show that TRAIL suppresses autoimmune damage in both relapsing-remitting and chronic non-remitting models of EAE. TRAIL-deficient mice and WT mice treated with neutralizing anti-mTRAIL antibody displayed increased clinical score, increased T cell proliferation responses to MOG, and increased numbers of inflammatory lesions in the CNS. Importantly, therapeutic intervention with recombinant soluble TRAIL/Apo2L or anti-TRAIL receptor (DR5) antibody reduced the severity of MOG-induced EAE. Together, these data illustrate the potential therapeutic value of rTRAIL/Apo2L and anti-DR5 antibody in suppressing EAE/MS and suggest that TRAIL may play a general role in suppression of T cell-mediated autoimmune diseases.

5.2 Introduction

MS is an autoimmune disease that involves both cellular and humoral responses directed against proteins of the myelin sheath, such as MOG, MBP and PLP (Abo et al., 1993; Mokhtarian et al., 1984; Schluesener et al., 1987; Steinman, 1996). The disease is characterized by an infiltration of inflammatory cells into the CNS, accompanied by localized destruction of the myelin sheath and loss of oligodendrocytes (Ewing and Bernard, 1998). MS lesions consist predominantly of activated $CD4^+$ T cells that secrete a variety of cytokines such as IFN- γ and TNF- α , B cells and macrophages and most patients have oligoclonal IgG bands with a specificity for myelin proteins in their cerebrospinal fluid (Ewing and Bernard, 1998; Raine, 1991). Most therapies for MS have focused on the use of steroids to suppress the inflammatory response associated with disease (Griffiths and Newman, 1994). Clinical trials using IFN- β have also yielded promising results, with IFN- β treatment decreasing the rate of relapse and delaying disability in patients (Pender and Wolfe, 2002; Ruuls and Sedgwick, 1998).

EAE is an animal model with many clinical and histopathological similarities to MS. Like MS, EAE is characterized by an early breach of the blood-brain barrier, infiltration of inflammatory cells into the CNS and demyelination that ultimately leads to paralysis of the animal (Bernard et al., 1992; Raine, 1990a). In mice, EAE can be induced by immunization with myelin or myelin components, including MBP, PLP or myelin peptides such as MOG. EAE is also induced by adoptive transfer of MBP-, PLP-, or MOG-specific $CD4^+$ Th1 T cells into naïve recipients. TCR transgenic mice that are specific for MBP, PLP and MOG have also been generated (Lafaille et al., 1994; Mendel et al., 2004; Waldner et al., 2000), although immunization is required to induce disease in some of these mice. MOG₃₅₋₅₅ peptide induces a relapsing-remitting form of EAE in NOD/Lt mice and a chronic non-remitting paralytic disease in C57BL/6 mice, which is characterized by MNC infiltration into the CNS and multifocal demyelination in the brain and spinal cord (Bernard et al., 1997; Slavin et al., 1998). The relapsing-remitting course of EAE that develops in NOD/Lt mice is thought to best approximate the MS disease profile in humans (Raine, 1990b).

TNF superfamily members play an important role in the regulation of a multitude of autoimmune diseases in humans and in animal models of autoimmune disease. Abnormal regulation of apoptosis, inflammation of the CNS and production of regulatory cytokines have been implicated in EAE. Members of the TNF superfamily play an important role in apoptosis, and many possess inflammatory activity that might influence the severity of MS/EAE. Moreover, cytokines produced during the course of disease (Ruuls and Sedgwick, 1998) may regulate the expression and function of TNF superfamily members. Within this family, TNF, FasL and LT- α have been examined in models of EAE, and each has been demonstrated to regulate disease (Dittel et al., 1999; Gommerman et al., 2003; Kassiotis and Kollias, 2001; Korner et al., 1997; Sabelko et al., 1997; Sabelko-Downes et al., 1999; Sean Riminton et al., 1998; Waldner et al., 1997). Recently, TRAIL was reported to suppress collagen-induced arthritis in DBA/1 mice (Song et al., 2000). TRAIL blockade using soluble TRAIL receptor (DR5) exacerbated disease score, increased lymphocyte proliferation within LNs, and increased both cytokine production and collagen-specific antibodies in mice immunized to develop disease (Song et al., 2000). Moreover, TRAIL gene transfer using a replication-defective adenovirus inhibited disease (Song et al., 2000).

As TRAIL was previously reported to inhibit collagen-induced arthritis (Song et al., 2000), we chose to investigate whether TRAIL plays a general role in suppression of T cell-mediated autoimmune diseases. In this study, we used TRAIL-deficient mice, a potent neutralizing anti-mTRAIL mAb, recombinant soluble TRAIL/Apo2L and an anti-TRAIL receptor (DR5) antibody to demonstrate that TRAIL suppresses autoimmune damage in chronic non-relapsing and relapsing-remitting models of EAE. We also show that TRAIL suppresses MOG-specific T cell proliferation responses and infiltration of inflammatory cells into the CNS in mice immunized to develop disease. As knowledge of the immunoregulatory processes involved in EAE increases, more specific intervention of disease in MS patients will become possible.

5.3 Materials and Methods

Mice

Inbred C57BL/6, BALB/c and NOD/Lt mice were purchased from The WEHI. C57BL/6 TRAIL^{-/-} and BALB/c TRAIL^{-/-} mice were obtained and bred at Peter Mac as detailed in Chapter 2, Section 2.1, Materials and Methods. Age matched 8-20 week old female mice were used in all experiments under specific pathogen-free conditions according to Animal Experimental Ethics Committee Guidelines and approval.

Reagents

Mouse MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) was purchased from Auspep (Parkville, Victoria, Australia) and determined to be over 94% pure by reversed-phase HPLC. Chicken OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) was kindly provided by Dr. Frank Carbone (University of Melbourne, Parkville, Victoria, Australia). Complete Freund's adjuvant (CFA) was purchased from GIBCO BRL (Rockville, MD, USA), *Mycobacterium tuberculosis* from Difco (Detroit, MI, USA), salt-free pertussis toxin from List Biological Laboratories (Campbell, CA, USA) and whole rat IgG from Sigma Chemical Co. The neutralizing anti-m TRAIL mAb (N2B2) was prepared as detailed in Chapter 2, Section 2.1, Materials and Methods and control rat IgG2a (R35-95) purchased from BD Biosciences Pharmingen. Anti-DR5 mAb (MD5-1) was kindly provided by Dr. Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan) and recombinant untagged soluble human Apo2L/TRAIL, currently being used in clinical trials, was provided by Dr. Avi Ashkenazi (Genentech Inc, South San Francisco, CA, USA) (Ashkenazi et al., 1999). Concanavalin A (Con A) was purchased from Sigma Chemical Co.

Induction and clinical evaluation of EAE

MOG₃₅₋₅₅ peptide (100 µg), emulsified in CFA and supplemented with *Mycobacterium tuberculosis* (4 mg/ml), was injected s.c. into each hind flank (total of 200 µg peptide per mouse). Shortly after, mice were injected i.p. with 350 ng pertussis toxin/PBS with a repeat dose 48 hours later (Bernard et al.,

1997). Mice were weighed three times weekly and monitored daily for clinical signs of disease. Neurological impairment was scored as follows: 0, no detectable impairment; 1, flaccid tail and/or abnormal gait; 2, hind limb weakness; 3, complete paralysis of one/both hind limbs; 4, complete hind limb paralysis and ascending paralysis; 5, moribund or deceased. In accordance with Animal Experimental Ethics Committee Guidelines, mice with a score of 4 or greater for more than 12 h were sacrificed. Mice that died due to severe EAE were scored as 5 for the remainder of the study.

Treatment with anti-mTRAIL mAb, anti-DR5 mAb and recombinant soluble TRAIL/Apo2L

To neutralize mouse TRAIL *in vivo*, mice were injected with between 250-500 µg anti-mTRAIL mAb (N2B2) three times weekly, commencing immediately prior to immunization, or on the days as indicated. Negative control mice were injected with either PBS or isotype control rat IgG. Administration of control IgG had no effect on disease course. To examine therapeutic activity, 50 µg recombinant soluble TRAIL in saline, or 50 µg anti-DR5 mAb was administered i.p. on the days as indicated. Anti-mTRAIL mAb (N2B2) does not neutralize human rTRAIL activity (data not shown).

CNS Histology

Brain and spinal cord were carefully dissected and immersion fixed or perfused with 4% paraformaldehyde in PBS. Fixed tissues were embedded in paraffin wax and 10 µm sections were cut from various locations of the brain and spinal cord. The preparation and staining of sections for histology was undertaken by technical staff at The WEHI. Sections were stained with H&E for evidence of inflammation (Johns et al., 1995).

³H-Thymidine cell proliferation assay

Splenocytes (1.5 to 4.5×10^5 /well) isolated from MOG₃₅₋₅₅ immunized mice were incubated in 96-well flat-bottomed plates with either 20 µg/ml MOG₃₅₋₅₅ peptide, 20 µg/ml OVA₂₅₇₋₂₆₄ peptide, or 3 µg/ml Con A (or the concentrations of peptide and Con A stated). Seventy-two hours later, ³H-thymidine (0.5 µCi) was

added and the cells were incubated for a further 18 h. Cells were harvested on glass fibre filter mats (Packard, Meriden, CT, USA), added to liquid scintillant (Perkin Elmer Life Sciences, Boston, MA, USA) and counted on a liquid scintillation analyzer (Tri-Carb2100TR, Packard).

Cytokine secretion ELISA

Splenocytes ($0.5\text{--}4.5 \times 10^5/\text{well}$) isolated from MOG₃₅₋₅₅ immunized mice were harvested at the end of the disease course and incubated in 96-well flat-bottomed plates in 20 $\mu\text{g}/\text{ml}$ MOG peptide, 3 $\mu\text{g}/\text{ml}$ Con A or media alone. Seventy-two hours later, supernatants were harvested and tested for cytokine concentration using the OptEIA IFN- γ ELISA kit according to the manufacturers' instructions (BD Biosciences Pharmingen).

MOG-specific antibody ELISA

Sera from MOG-immunized mice were tested for MOG-reactive antibodies by ELISA. Ninety-six-well plates were pre-coated with 5 $\mu\text{g}/\text{ml}$ MOG peptide in carbonate buffer pH 9.6, overnight at 4°C. Plates were then washed 3-5 times with 0.05% Tween-20/PBS and blocked for 1.5 h at RT with 2% BSA/PBS. Subsequent steps with intervening washes included adding sera for 2 h at RT, HRP-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 2 h at RT and development with *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.) in phosphate citrate buffer (pH 5.0) for 30 min. Reactions were stopped using 3 M HCl and read at OD 492 nm.

Statistical analysis.

Data were analyzed using a two tailed, non-parametric, unpaired Mann Whitney test. P values < 0.05 were considered significant.

5.4 Results

5.4.1 TRAIL neutralization exacerbates relapsing-remitting and chronic non-remitting EAE

The relapsing-remitting course of EAE that develops in MOG₃₅₋₅₅ peptide immunized NOD/Lt mice is thought to best approximate the MS disease profile in humans (Raine, 1990b). To investigate the role of endogenous TRAIL in autoimmune-mediated demyelination, NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE. Mice were injected 3 times weekly, beginning immediately prior to immunization, with an anti-mTRAIL mAb previously demonstrated to effectively block TRAIL function *in vivo* (Smyth et al., 2001; Takeda et al., 2001; Takeda et al., 2002). Mean clinical score was greater for mice treated with anti-mTRAIL mAb, and these mice developed a severe progressive disease, compared with control treated mice that developed the usual relapsing-remitting disease that is characterized by 'peaks' and 'troughs' in disease score (Figure 5.1A). In addition, mean cumulative score was significantly higher, and mean day of disease onset significantly earlier for anti-mTRAIL mAb-treated mice compared to control-treated NOD/Lt mice ($P < 0.05$, Table 5.1). Moreover, 60% of anti-mTRAIL mAb-treated NOD/Lt mice died during the course the study, compared to 20% of control-treated mice (Table 5.1).

To investigate whether endogenous TRAIL also suppresses chronic non-remitting EAE, C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE and injected 3 times weekly, beginning immediately prior to immunization, with anti-mTRAIL mAb. Clinical symptoms were exacerbated in mice treated with anti-mTRAIL mAb compared to isotype control-treated mice, particularly at the later stages of disease (25 days post immunization onward) (Figure 5.1B). Moreover, mean maximum score was significantly higher for anti-mTRAIL mAb-, compared to isotype control-treated C57BL/6 mice ($P < 0.05$), and 100% of anti-mTRAIL mAb-treated C57BL/6 mice died during the course of the study, compared to 0% of control-treated mice (Table 5.1). Together, these data

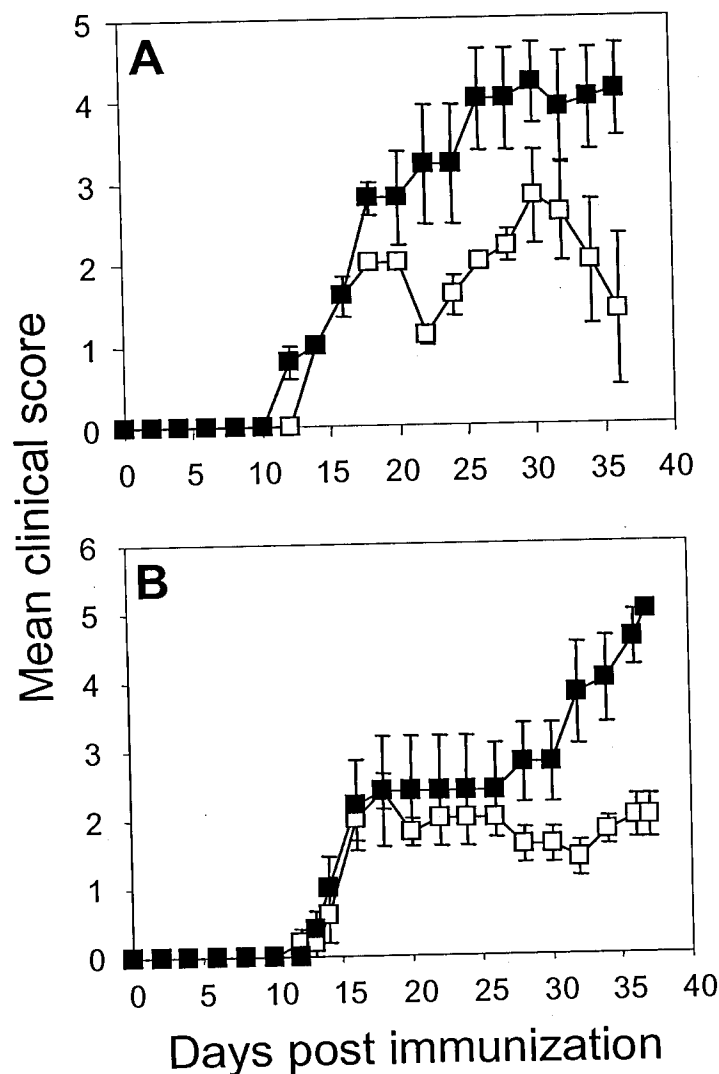


Figure 5.1. Host TRAIL suppresses relapsing-remitting and non-relapsing EAE. (A) Relapsing-remitting EAE: female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide and injected three times weekly for the duration of the experiment with either PBS (open squares, n=5) or 250 μ g anti-mTRAIL mAb i.p. (closed squares, n=5) to develop EAE. (B) Chronic non-relapsing EAE: female C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide and injected three times weekly for the duration of the experiment with either 500 μ g rat IgG (open squares, n=5) or 500 μ g anti-mTRAIL mAb i.p. (closed squares, n=5) to develop EAE. Mean \pm SEM are shown for each time point. Results are representative of 4 independent experiments in (A) and 1 in (B).

Figure	Strain	Treatment	Incidence	Deaths	Mean day of onset \pm SEM	Mean max score ¹ \pm SEM	Mean cumulative score ² \pm SEM (days scored)
5.1A	NOD/Lt	PBS	5/5	1/5	13.6 \pm 0.2	2.80 \pm 0.58	43.7 \pm 5.8 (36)
"	NOD/Lt	Anti-TRAIL	5/5	3/5	11.8 \pm 0.4*	4.20 \pm 0.49	77.2 \pm 10.2 (36)*
5.1B	C57BL/6	Control IgG	5/5	0/5	14.4 \pm 0.7	2.80 \pm 0.20	43.4 \pm 0.8 (37)
"	C57BL/6	Anti-TRAIL	5/5	5/5	16.0 \pm 2.3	5.00 \pm 0.00*	69.0 \pm 13.6 (37)
5.2	C57BL/6		7/7	0/7	16.6 \pm 1.4	2.14 \pm 0.26	29.3 \pm 5.7 (40)
"	C57BL/6 TRAIL ^{-/-}		7/7	1/7	13.7 \pm 2.2	3.43 \pm 0.43*	71.4 \pm 12.4 (40)*
5.10 A	NOD/Lt	Control Ig	6/6	0/6	13.3 \pm 1.2	2.50 \pm 0.34	45.3 \pm 5.3 (40)
"	NOD/Lt	rTRAIL	6/6	0/6	27.0 \pm 2.7*	1.17 \pm 0.17*	13.3 \pm 3.0 (40)*
5.10 B	NOD/Lt	Anti-TRAIL, Control Ig	6/6	1/6	13.6 \pm 0.7	3.67 \pm 0.33	62.5 \pm 5.3 (40)
"	NOD/Lt	Anti-TRAIL, rTRAIL	6/6	0/6	23.7 \pm 2.9*	1.67 \pm 0.21*	17.8 \pm 3.4 (40)*
5.12	NOD/Lt	Control IgG	6/6	1/6	15.7 \pm 1.4	3.17 \pm 0.48	56.0 \pm 9.0 (40)
"	NOD/Lt	Anti-DR5	5/6	0/6	23.6 \pm 4.0 ³	1.33 \pm 0.43*	15.2 \pm 5.1 (40)*
"	NOD/Lt	rTRAIL	6/6	0/6	19.0 \pm 2.7	1.33 \pm 0.21*	22.2 \pm 4.2 (40)*

Table 5.1. Effect of TRAIL on clinical parameters following MOG immunization. ¹Mean maximum (max) score was calculated from the highest score achieved for each individual mouse in a group over the course of disease. ²Mean cumulative score was calculated from the sum of the scores for each day from day 0 for the period of days indicated in parentheses. ³ Mean day of onset was calculated from the day of onset of the 5 mice in this group that succumbed to disease. Disease was completely suppressed in one mouse. * P < 0.05 compared to group above, except last row which is compared to control IgG group 2 rows above.

suggested that endogenous TRAIL suppressed both relapsing-remitting and chronic non-remitting forms of EAE.

5.4.2 TRAIL-deficient C57BL/6 mice develop exacerbated EAE

To examine the effect of TRAIL-deficiency on development of chronic non-remitting EAE, C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE. Similar to anti-mTRAIL mAb-treated C57BL/6 mice, C57BL/6 TRAIL^{-/-} mice developed exacerbated EAE compared to C57BL/6 WT control mice, particularly at the later stages of disease (24 days post immunization onward) (Figure 5.2). Mean maximum score and mean cumulative score were also significantly higher for C57BL/6 TRAIL^{-/-} compared to C57BL/6 WT mice (Table 5.1, $P < 0.05$). Fourteen percent of C57BL/6 TRAIL^{-/-} mice died during the course of the study, compared to 0% of C57BL/6 WT mice (Table 5.1). These data further confirmed that endogenous TRAIL suppressed chronic non-remitting EAE.

5.4.3 TRAIL neutralization induces EAE in 'resistant' BALB/c mice

We then explored the possibility that endogenous TRAIL suppresses EAE in BALB/c mice that are typically resistant to disease development. BALB/c mice were immunized with MOG₃₅₋₅₅ peptide and injected 3 times weekly with anti-mTRAIL mAb, commencing immediately prior to immunization. BALB/c mice immunized with MOG₃₅₋₅₅ peptide and injected with isotype control mAb were completely resistant to disease induction (Figure 5.3A). In contrast, resistance was overcome in 2/6 BALB/c mice injected with anti-mTRAIL mAb, with one of these mice developing a severe form of EAE and achieving a clinical score of 3 (Figure 5.3B). This suggested that TRAIL contributed to the resistance of BALB/c mice to EAE.

5.4.4 EAE is induced in TRAIL-deficient BALB/c mice

To examine the effect of TRAIL-deficiency on disease induction in typically resistant BALB/c WT mice, BALB/c WT and BALB/c TRAIL^{-/-} mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE. BALB/c WT mice immunized

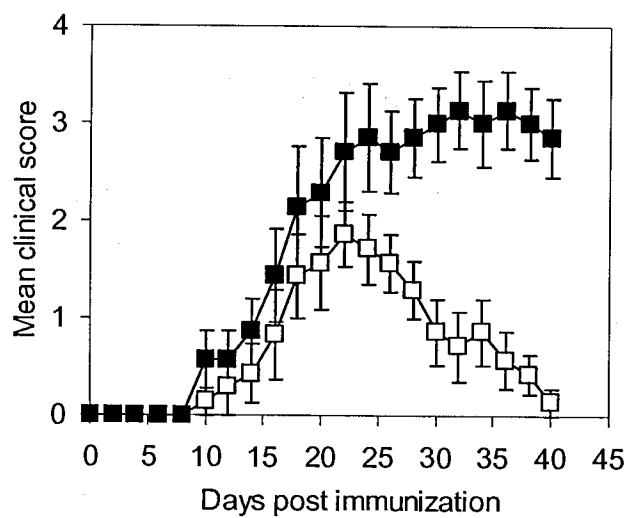


Figure 5.2. C57BL/6 TRAIL^{-/-} mice develop exacerbated EAE. Female C57BL/6 (open squares, n=7) and C57BL/6 TRAIL^{-/-} mice (closed squares, n=7) were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mean \pm SEM are shown for each time point. Results are representative of 4 independent experiments.

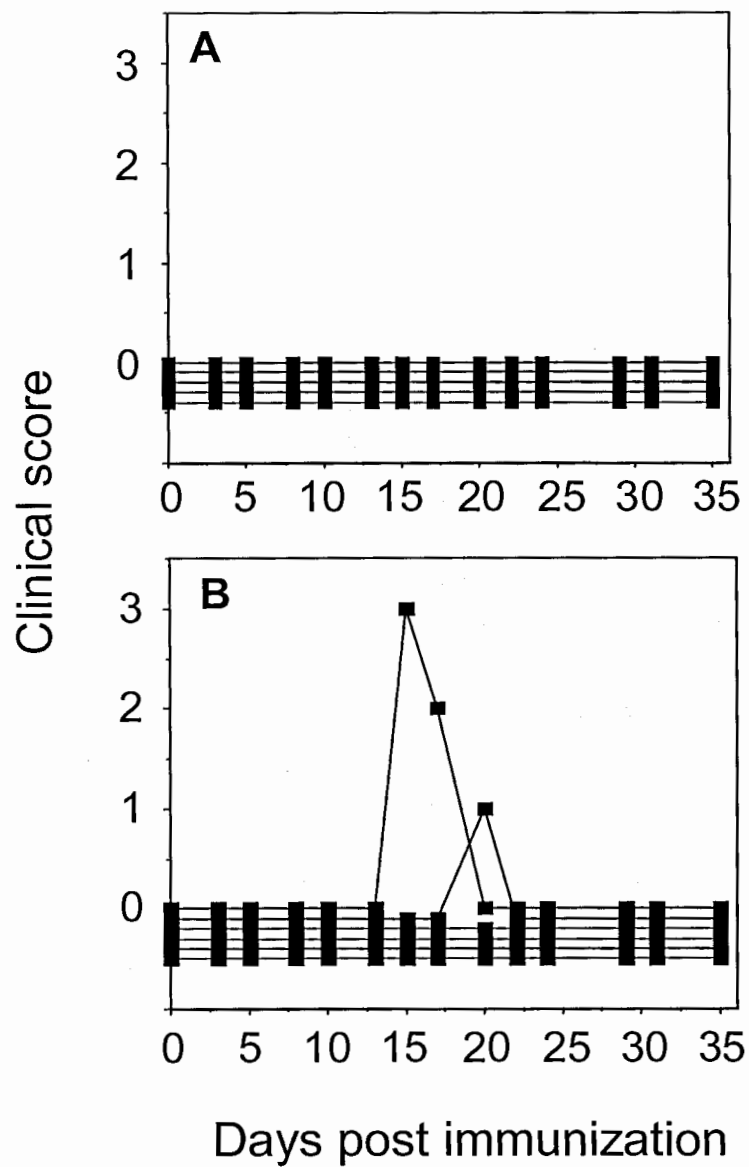


Figure 5.3. Host TRAIL suppresses EAE in 'resistant' BALB/c mice. Female BALB/c mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE and injected i.p. three times weekly from the day of immunization, for the duration of the experiment, with either 500 µg rat IgG (panel A, n=5) or 500 µg anti-mTRAIL mAb (panel B, n=6). Individual lines represent the disease course of each mouse.

with MOG₃₅₋₅₅ peptide were largely resistant to disease induction, although 1/5 mice developed a limp tail (clinical score of 1) for a period of 24 h (Figure 5.4A). In contrast, resistance was overcome in 3/6 BALB/c TRAIL^{-/-} mice, with one of these mice developing a severe form of EAE and achieving a clinical score of 3 (Figure 5.4B). These data further supported the contention that TRAIL contributed to the resistance of BALB/c WT mice to EAE.

5.4.5 TRAIL suppresses T cell infiltration and inflammation in the central nervous system (CNS)

Like MS, EAE is typically characterized by an infiltration of inflammatory cells into the CNS (Bernard et al., 1992; Raine, 1990a). To evaluate whether TRAIL regulated inflammation associated with EAE, NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE and injected 3 times weekly with anti-mTRAIL mAb or PBS. At the termination of the experiment (day 36), CNS sections from these mice were analyzed. Far more extensive inflammation and greater numbers of lesions were revealed in the CNS parenchyma (Figure 5.5), cerebellum (Figure 5.6) and spinal cord (Figure 5.7) of anti-mTRAIL mAb-treated, compared with PBS-treated mice. Moreover, extensive oedema was observed in the spinal cord of anti-mTRAIL mAb-treated, compared to control-treated mice (Figure 5.7). These data demonstrated that TRAIL suppressed EAE by inhibiting the infiltration of inflammatory cells into the CNS and by inhibiting oedema associated with disease.

5.4.6 TRAIL suppresses T cell proliferation but has no effect on MOG-specific antibody or cytokine production

To determine whether TRAIL influences the autoreactive phase of EAE, we examined the immune response generated against MOG. At the termination of the experiment (day 36), splenocytes isolated from MOG₃₅₋₅₅ peptide-immunized NOD/Lt mice were restimulated with MOG₃₅₋₅₅ peptide *in vitro* and MOG-specific proliferation was determined by ³H-thymidine incorporation assay. MOG-specific proliferative responses were almost 3-fold higher for splenocytes isolated from anti-mTRAIL mAb-treated, compared with control-treated NOD/Lt mice (Figure 5.8A), suggesting that TRAIL inhibited lymphocyte proliferation

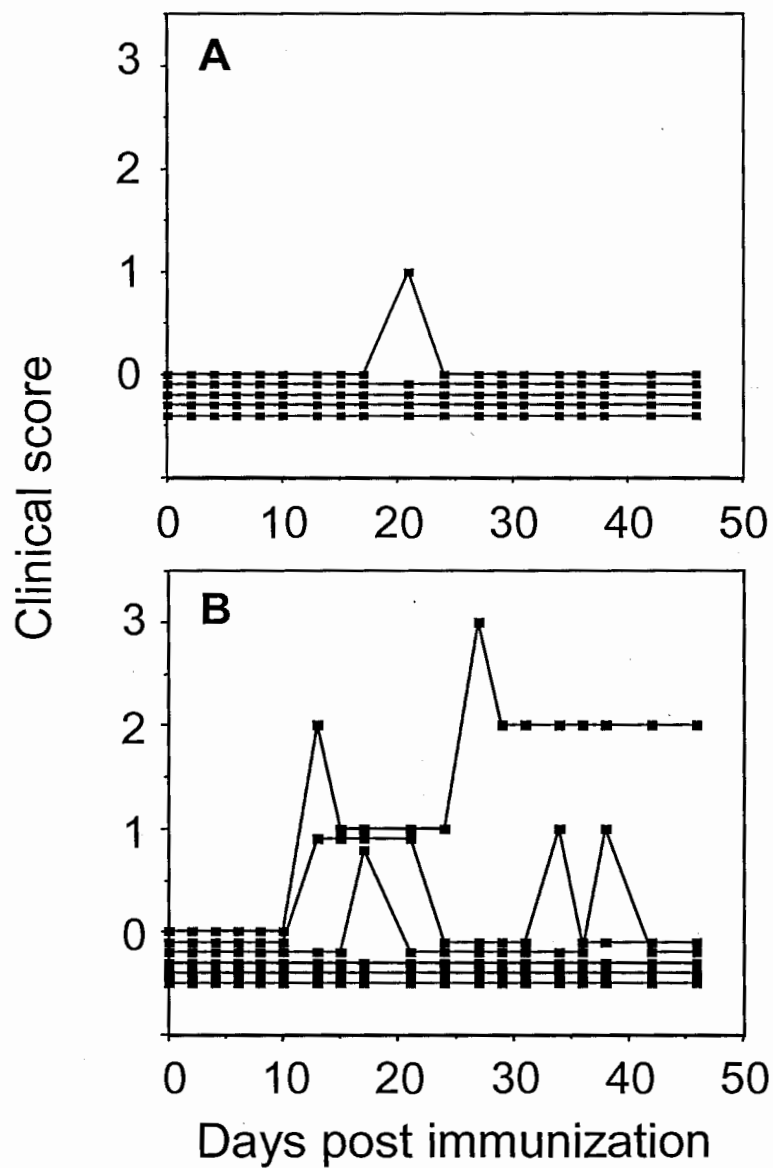


Figure 5.4. Resistance to EAE is overcome in BALB/c TRAIL^{-/-} mice. Female BALB/c (panel A, n=5) and BALB/c TRAIL^{-/-} (panel B, n=6) mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Individual lines represent the disease course of each mouse. Results are representative of 3 independent experiments.

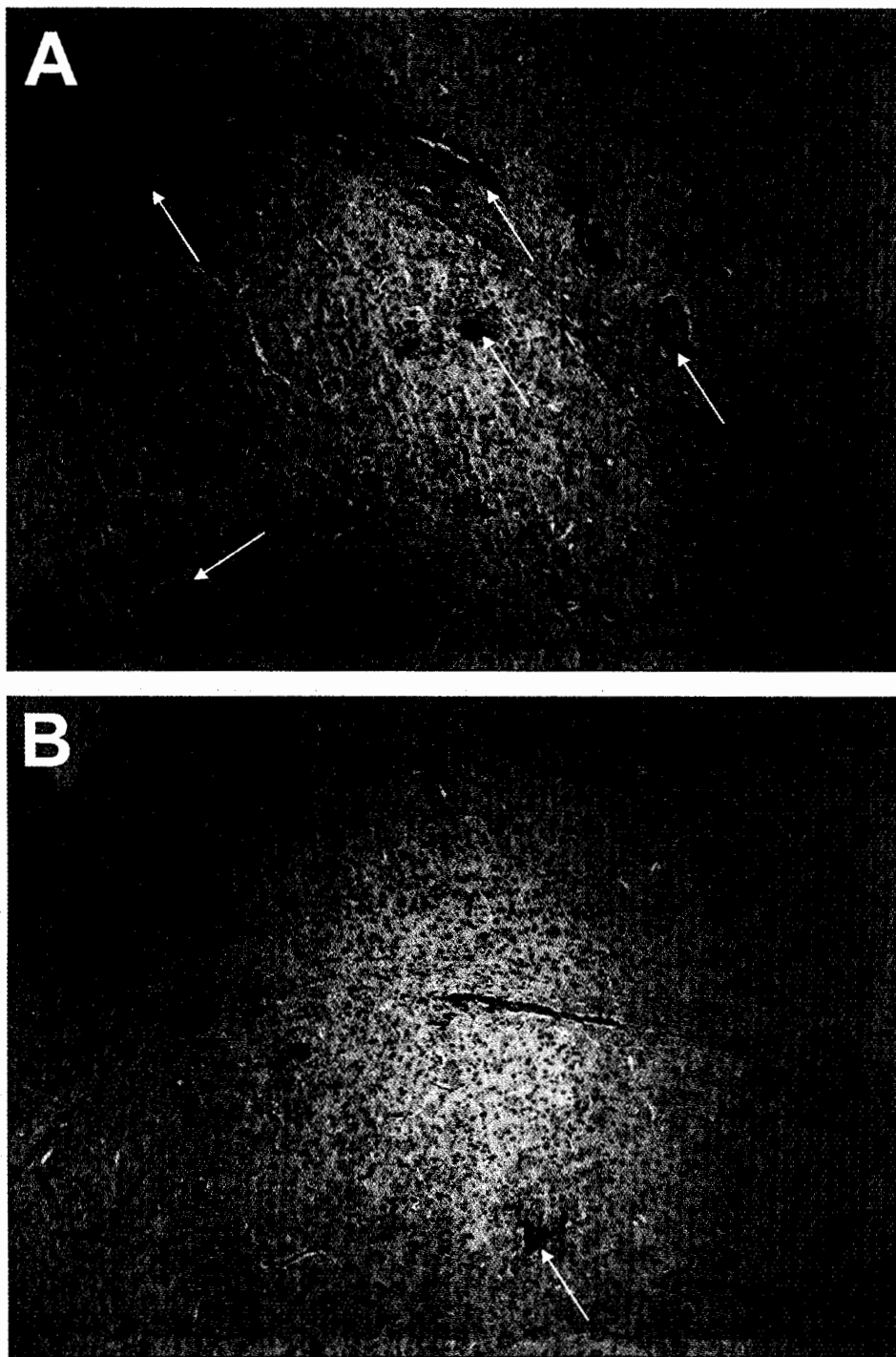


Figure 5.5. TRAIL suppresses leukocyte cell infiltration in the CNS parenchyma. Female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected i.p. three times weekly from the day of immunization, for the duration of the experiment, with 500 μ g anti-mTRAIL mAb (A) or PBS (B). Thirty-six days after immunization, mice were sacrificed and H&E staining performed on CNS parenchyma (original magnification x 10). White arrows indicate inflammatory lesions. Histology is representative of 5 NOD/Lt mice for each group.

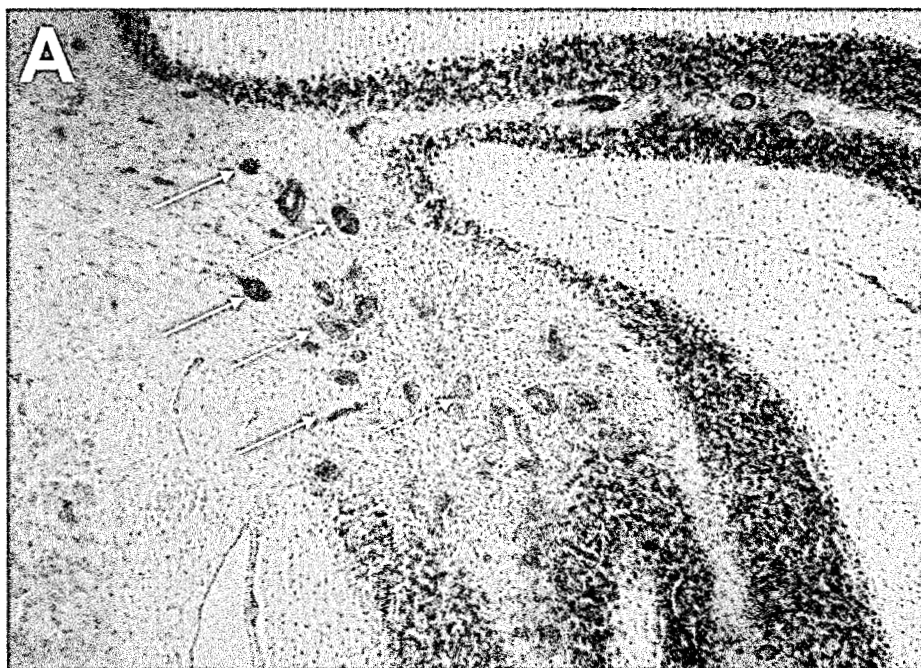


Figure 5.6. TRAIL suppresses leukocyte cell infiltration in the cerebellum. Female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected i.p. three times weekly from the day of immunization, for the duration of the experiment, with 500 μ g anti-mTRAIL mAb (A) or PBS (B). Thirty-six days after immunization, mice were sacrificed and H&E staining performed on the cerebellum (original magnification x 10). White arrows indicate inflammatory lesions. Histology is representative of 5 NOD/Lt mice for each group.

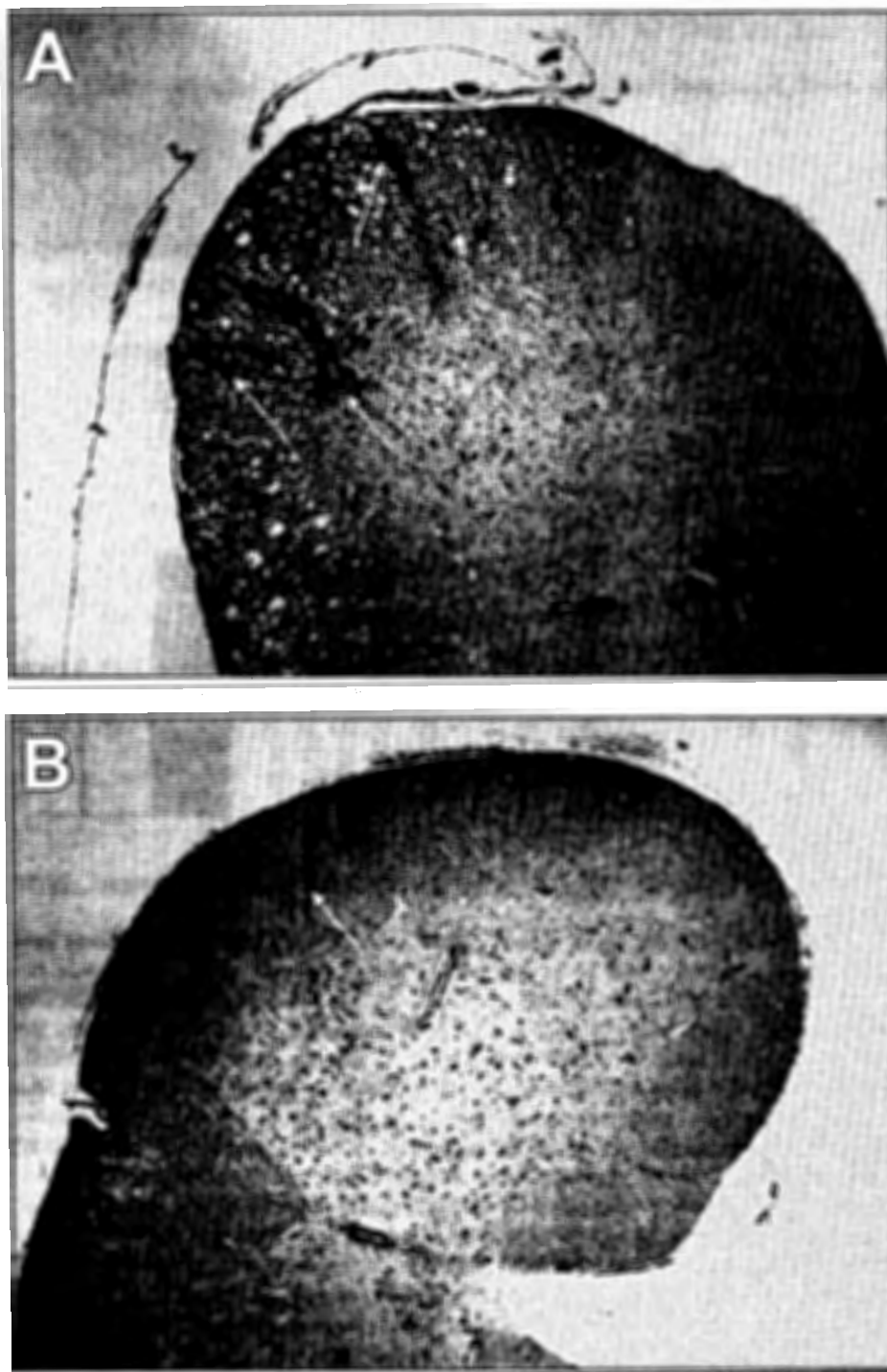


Figure 5.7. TRAIL suppresses leukocyte cell infiltration and oedema in the spinal cord. Female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected i.p. three times weekly from the day of immunization, for the duration of the experiment, with 500 µg anti-mTRAIL mAb (A) or PBS (B). Thirty-six days after immunization, mice were sacrificed and H&E staining performed on spinal cord (original magnification x 10). White arrows indicate inflammatory lesions, black arrows indicate the presence of oedema. Histology is representative of 5 NOD/Lt mice for each group.

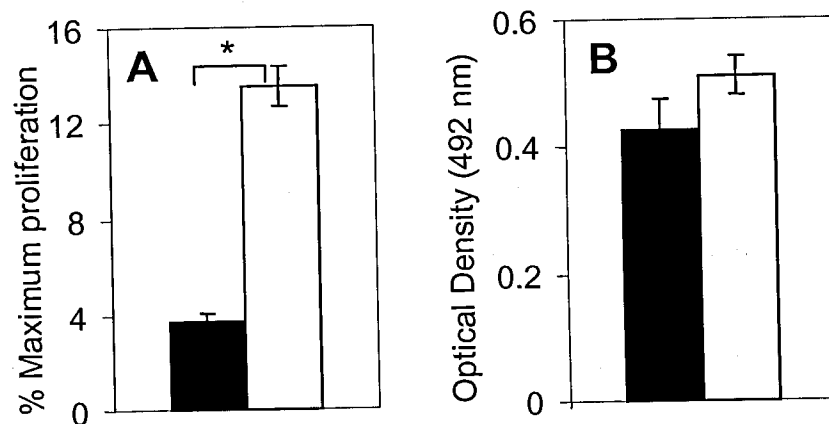


Figure 5.8. TRAIL inhibits MOG-specific T cell proliferation, but not antibody production in NOD/Lt mice. Female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE and injected i.p. three times weekly from the day of immunization, for the duration of the experiment, with either control PBS or 500 µg anti-mTRAIL mAb. Thirty-six days after immunization, mice were sacrificed and their splenocytes analyzed for (A) MOG-specific proliferation. (B) Serum isolated from the same mice was tested for MOG-specific antibody production by ELISA (measured by a change in absorbance at 492 nm). Black columns represent NOD/Lt mice injected with PBS and white columns represent NOD/Lt mice injected with anti-mTRAIL mAb. Percentage maximum proliferation = (MOG proliferation – media proliferation) / (Con A proliferation – media proliferation) × 100%. Mean ± SEM were calculated from the analysis of 4 NOD/Lt mice treated with PBS and 4 NOD/Lt mice treated with anti-mTRAIL mAb. Results are representative of 3 independent experiments for (A) and 2 independent experiments for (B). Asterisk indicates that proliferation is significantly different between NOD/Lt mice injected with PBS and NOD/Lt mice injected with anti-mTRAIL mAb (* P < 0.03).

associated with relapsing-remitting EAE. In contrast, TRAIL had no discernable affect on the levels of total MOG-reactive antibody in the serum of these mice (Figure 5.8B).

We then examined MOG-specific proliferative responses in C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice. At the termination of the experiment (day 35), splenocytes isolated from MOG₃₅₋₅₅ peptide immunized mice were restimulated with peptide *in vitro*, and the MOG-specific proliferation response determined by ³H-thymidine assay. MOG-specific proliferative responses were at least 3-fold higher for splenocytes isolated from C57BL/6 TRAIL^{-/-} mice compared with C57BL/6 WT mice (Figure 5.9A), suggesting that TRAIL inhibited lymphocyte proliferation associated with chronic non-relapsing EAE. Restimulated splenocytes from C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice were also examined for MOG-specific production of IFN- γ *in vitro* (Figure 5.9B), although TRAIL-deficiency appeared to have no affect on cytokine production *in vitro*.

5.4.7 rTRAIL/Apo2L suppresses EAE in non-obese diabetic (NOD)/Lt mice

In murine models, recombinant soluble human TRAIL/Apo2L (rTRAIL/Apo2L) has demonstrated remarkable anti-tumor activity against human colon carcinoma (Ashkenazi et al., 1999; LeBlanc et al., 2002), glioma (Fulda et al., 2002; Pollack et al., 2001), multiple myeloma (Mitsiades et al., 2001) and breast carcinoma xenografts (Walczak et al., 1999). As we had demonstrated a role for endogenous TRAIL in suppression of EAE in mice of three different genetic backgrounds, we assessed whether continuous treatment with human rTRAIL/Apo2L could suppress relapsing-remitting EAE in MOG₃₅₋₅₅ peptide-immunized NOD/Lt mice. Whilst NOD/Lt mice immunized to develop EAE and treated with control Ig developed the usual relapsing-remitting disease, treatment with rTRAIL/Apo2L dramatically decreased clinical signs of disease (Figure 5.10A). Mean maximum score and mean cumulative score were significantly decreased and mean day of disease onset was significantly later in rTRAIL/Apo2L-treated mice ($P < 0.05$, Table 5.1). The therapeutic efficacy of rTRAIL/Apo2L was also tested in NOD/Lt mice stimulated with anti-mTRAIL mAb to develop an exacerbated form of EAE. Treatment with rTRAIL/Apo2L also dramatically decreased the

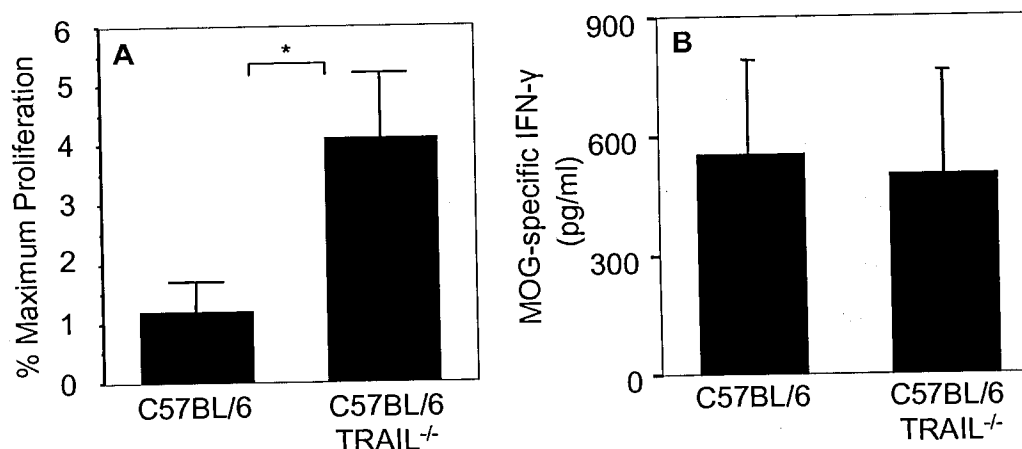


Figure 5.9. TRAIL inhibits MOG-specific T cell proliferation, but not IFN- γ production in C57BL/6 mice. Female C57BL/6 or C57BL/6 TRAIL^{-/-} mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Thirty-five days after immunization, mice were sacrificed and their splenocytes tested for (A) MOG peptide-specific proliferation, or (B) MOG-specific IFN- γ production. Percentage maximum proliferation = (MOG proliferation – media proliferation) / (Con A proliferation – media proliferation) \times 100%. MOG-specific IFN- γ production = IFN- γ produced in the presence of MOG – IFN- γ produced in the absence of MOG. Mean \pm SEM were calculated from the analysis of 6 mice for each group tested. Asterisk indicates that proliferation is significantly different between C57BL/6 and C57BL/6 TRAIL^{-/-} mice (* $P < 0.03$). Results are representative of 2 independent experiments.

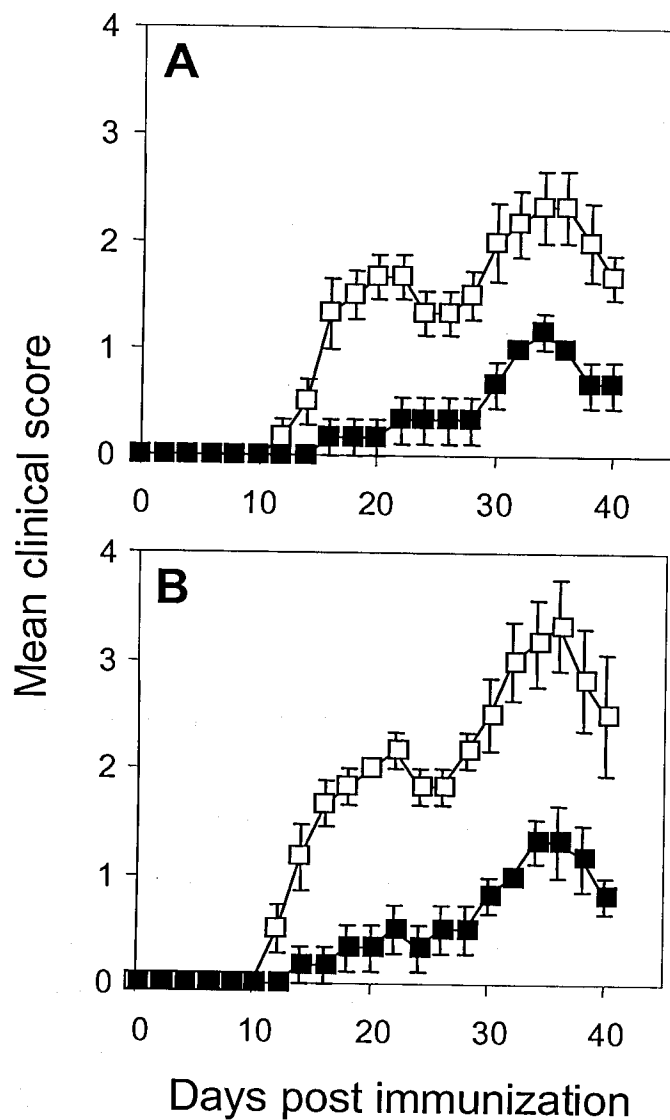


Figure 5.10. rTRAIL/Apo2L suppresses EAE. (A) Female NOD/Lt WT mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected on days 0, 2, 4, 7, 9, 11, 14, 16, 20, 22, 24, 28, 30 and 36 with either 50 µg control Ig (open symbols, n = 6) or 50 µg recombinant human TRAIL/Apo2L (closed symbols, n = 6). (B) Female NOD/Lt WT mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. All mice were injected on days 0, 2, 4, 7, 9, 11, 14, 16, 20, 22, 24, 28, 30 and 36 with 250 µg anti-mTRAIL mAb to develop exacerbated EAE. Mice were concurrently injected on days 0, 2, 4, 7, 9, 11, 14, 16, 20, 22, 24, 28, 30 and 36 with either 50 µg control Ig (open symbols, n = 6) or 50 µg rTRAIL/Apo2L (closed symbols, n = 6). Mean \pm SEM are shown for each time point. Results are representative of 2 independent experiments.

severity of disease in these mice, suggesting that rTRAIL/Apo2L treatment was an effective means of suppressing acute EAE (Figure 5.10B, Table 5.1).

As we had shown that endogenous TRAIL suppresses T cell proliferation associated with EAE, we examined whether rTRAIL/Apo2L treatment suppressed EAE by inhibiting MOG-specific T cell proliferative responses in mice. At the termination of the experiment (day 40), splenocytes from MOG₃₅₋₅₅ peptide immunized mice were restimulated with peptide *in vitro* and MOG-specific proliferation was examined. As shown previously (Figure 5.8A), MOG-specific proliferative responses were 2-4-fold higher in anti-mTRAIL mAb-treated, compared to control mAb-treated NOD/Lt mice (Figure 5.11). In contrast, MOG-specific proliferation was suppressed in rTRAIL/Apo2L-treated mice (Figure 5.11). These data suggested that like endogenous TRAIL, rTRAIL/Apo2L inhibited EAE in NOD/Lt mice by suppressing MOG-specific T cell proliferation.

5.4.8 Anti-DR5 monoclonal antibody (mAb) suppresses EAE in NOD/Lt mice

In mice, anti-mouse TRAIL receptor (DR5) mAb has been reported to induce potent anti-tumor effects against TRAIL-sensitive tumor cells by recruiting Fc-receptor-expressing innate immune cells such as NK cells and macrophages (Takeda et al., 2004). Given that rTRAIL/Apo2L potently suppressed EAE, we then assessed whether continuous treatment with anti-DR5 mAb could suppress EAE. NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE and injected three times weekly with anti-DR5 mAb, commencing immediately prior to immunization. Clinical signs of disease were dramatically decreased in anti-DR5 mAb-treated mice, compared with control-treated mice (Figure 5.12). Mean maximum score and mean cumulative score were also significantly decreased in anti-DR5-treated NOD/Lt mice, compared to control-treated mice ($P < 0.05$, Table 5.1). The extent of disease inhibition afforded by anti-DR5 mAb therapy was equivalent to that achieved by treatment with rTRAIL/Apo2L (Figure 5.12). These data collectively demonstrated the therapeutic efficacy of rTRAIL/Apo2L and anti-DR5 mAb in suppression of EAE.

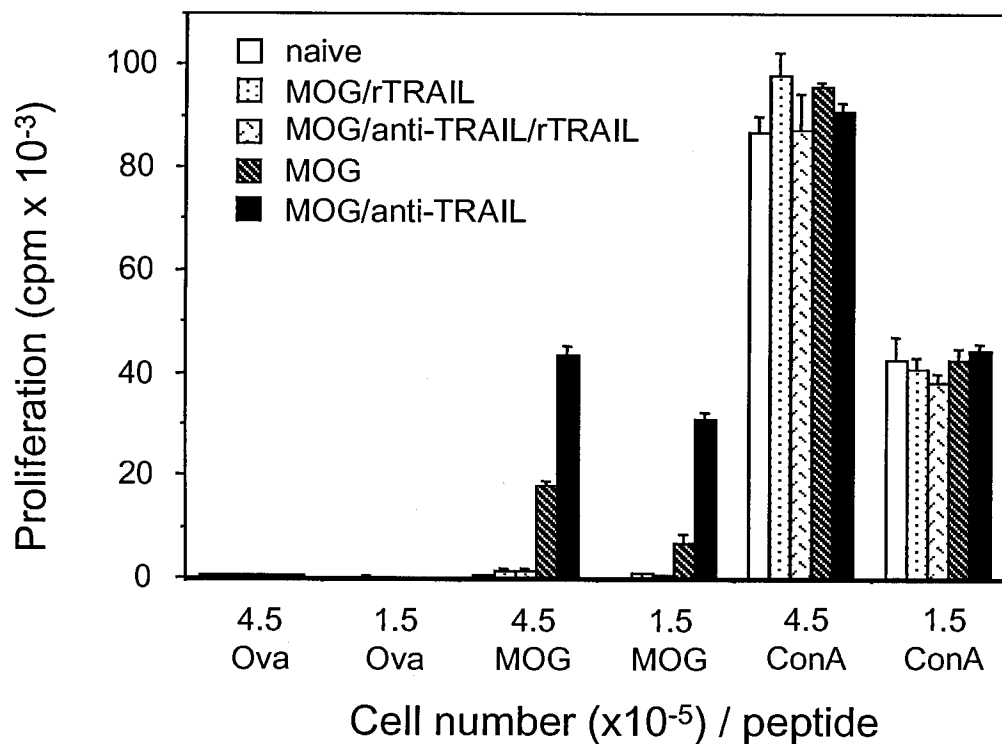


Figure 5.11. rTRAIL/Apo2L suppresses MOG-specific T cell proliferation. Female NOD/Lt WT mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected on days 0, 2, 4, 7, 9, 11, 14, 16, 20, 22, 24, 28, 30 and 36 with either 50 μ g control Ig (n = 6) or 50 μ g rTRAIL/Apo2L (n = 6). Some mice were additionally injected i.p. on days 0, 2, 4, 7, 9, 11, 14, 16, 20, 22, 24, 28, 30 and 36 with 250 μ g anti-mTRAIL mAb (n=6) to develop exacerbated EAE. Forty days after immunization, mice were sacrificed, their splenocytes pooled and tested for MOG₃₅₋₅₅ peptide-specific proliferation. Proliferation in the presence of Con A or OVA₂₅₇₋₂₆₄ peptide was tested as a positive and negative control. Proliferation of naïve splenocytes is additionally shown as a negative control. Results are expressed as the mean \pm SEM of cpm calculated from the analysis of triplicate samples.

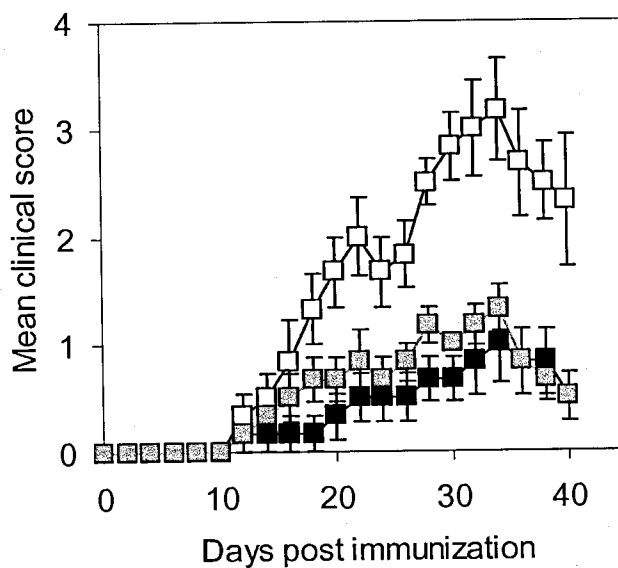


Figure 5.12. Anti-DR5 mAb treatment suppresses EAE. Female NOD/Lt mice were immunized with MOG₃₅₋₅₅ peptide to develop EAE. Mice were injected every second day with either 50 µg control Ig (open squares), 50 µg rTRAIL/Apo2L (grey squares) or 250 µg anti-DR5 mAb (black squares). Mean \pm SEM were calculated from clinical scores determined for n=6 mice for each group.

5.5 Discussion

A number of studies have demonstrated the selective ability of rTRAIL to induce apoptosis in transformed and virus-infected cells, but not in most normal cells (Ashkenazi et al., 1999; Walczak et al., 1999). Moreover, in Chapter 3, we reported that TRAIL suppressed tumor initiation, growth and metastases *in vivo*. Whilst a role for TRAIL in transformed and virus-infected tissues has been clearly established, the function of TRAIL in non-transformed tissues was largely unknown. We have now demonstrated a role for endogenous TRAIL in the suppression of both relapsing-remitting and chronic non-remitting EAE. In addition, we have shown that TRAIL contributes to the resistance of BALB/c mice to induction of EAE. Recall of T cell proliferation responses to MOG autoantigen was greater in TRAIL-deficient mice or WT mice treated with neutralizing anti-mTRAIL mAb, compared with WT mice. Mice lacking TRAIL also exhibited increased leukocyte infiltration into the CNS compared to WT mice. Importantly, this is the first report to demonstrate the therapeutic efficacy of exogenously administered rTRAIL/Apo2L or anti-DR5 mAb in suppression of EAE. Together, our findings suggested that recombinant human TRAIL/Apo2L or humanized anti-DR5 mAb administration may be effective in suppressing disease episodes in MS patients.

During the course of our studies, TRAIL was demonstrated by others to suppress both cyclophosphamide- and streptozotocin-induced diabetes in mice (Lamhamedi-Cherradi et al., 2003a; Lamhamedi-Cherradi et al., 2003b; Mi et al., 2003). Furthermore, TRAIL-deficient mice were reported to be hypersensitive to collagen-induced arthritis (Lamhamedi-Cherradi et al., 2003b) and TRAIL blockade using soluble TRAIL receptor (DR5) exacerbated MOG-induced EAE in C57BL/6 WT mice (Hilliard et al., 2001). Whilst the role of many of the TNF superfamily members in autoimmune disease has been controversial, TRAIL has consistently been shown to inhibit autoimmune disease induction. These recent findings, combined with our results, suggest an important and general role for TRAIL in suppression of T-cell mediated autoimmune disease.

Although the precise mechanism(s) whereby TRAIL inhibits EAE were not clear, our data suggested that TRAIL inhibited T cell proliferation and infiltration of leukocytes into the CNS. Our data also suggested that TRAIL has no effect on MOG-specific cytokine or antibody production in EAE. Hilliard and colleagues similarly reported that TRAIL suppressed leukocyte infiltration into the CNS, but in contrast to our findings, they reported that TRAIL additionally inhibits cytokine production associated with disease (Hilliard et al., 2001). Importantly, MOG-specific cytokine production was tested at an earlier stage of disease (day 26 compared to day 35 in our study), and TRAIL activity was only blocked for the first 16 days of the experiment in the Hilliard et al. study, whilst we used TRAIL-deficient mice. Perhaps these differences account for the discrepancies in our findings. Further studies are clearly required to determine the exact mechanism(s) by which TRAIL suppresses EAE, and the key TRAIL-expressing effector cells that are involved.

TRAIL is constitutively expressed on a proportion of mouse liver NK cells (Takeda et al., 2001), and TRAIL expression can be induced on NK cells, T cells, monocytes and DCs upon stimulation with interferons or cytokines such as IL-2 and IL-15 (Fanger et al., 1999; Griffith et al., 1999; Kayagaki et al., 1999a; Kayagaki et al., 1999b; Zamai et al., 1998). IFN- β has recently been shown to be effective in the treatment of MS and EAE (Pender and Wolfe, 2002; Ruuls and Sedgwick, 1998; Teige et al., 2003; van der Meide et al., 1998; Wender et al., 2001; Yasuda et al., 1999; Yu et al., 1996), with several studies suggesting that IFN- β inhibits pro-inflammatory cytokines and stimulates anti-inflammatory cytokines to regulate disease (Teige et al., 2003; Wender et al., 2001; Yasuda et al., 1999). As IFN- β is one of the best stimuli of NK cell TRAIL expression (Sato et al., 2001), and NK cells can inhibit EAE (Zhang et al., 1997), IFN- β might suppress MS/EAE by stimulating a population of effector NK cells to express TRAIL. TRAIL-expressing NK cells may be hypothesized to inhibit EAE by inducing apoptosis in autoreactive T cells or DCs, thus preventing autoimmune damage and the further priming of autoreactive T cells. IFN- β is also known to upregulate TRAIL expression on DCs (Liu et al., 2001) and these DCs may be hypothesized to suppress disease by deleting autoreactive T cells. Indeed,

recently, DCs have been reported to inhibit the proliferation of autoreactive T cells in EAE in a TRAIL-dependent manner, suggesting an alternative role for DCs in regulation of EAE (Suter et al., 2003).

Recently, a critical role for TRAIL in negative selection was proposed by Lamhamedi-Cherradi and colleagues (Lamhamedi-Cherradi et al., 2003b). These authors postulated, but did not directly demonstrate, that impaired negative selection of T cells in TRAIL-deficient mice may lead to enhanced susceptibility to autoimmune disease. This report clearly contrasted with a previous study that used soluble blocking TRAIL-R2-Fc to demonstrate that negative selection of mouse and human thymocytes was TRAIL-independent (Simon et al., 2001), and studies that excluded a role for the FADD-caspase 8 pathway (Newton et al., 1998; Smith et al., 1996). In Chapter 6, we show that TRAIL does not play a role in thymocyte negative selection, suggesting that TRAIL regulates EAE induction by a different mechanism. TRAIL is known to contribute to AICD of PBLs, *in vitro* activated human CD4⁺ T cell clones, and the human leukemia line Jurkat (Martinez-Lorenzo et al., 1998; Zhang et al., 2003). It remains to be determined whether potentially-autoreactive T cells in the periphery undergo AICD by a TRAIL-dependent mechanism. Understanding how TRAIL suppresses EAE will be of great importance prior to any future planning of clinical trials in MS patients using rTRAIL/Apo2L.

While TRAIL-deficient mice and mice in which TRAIL activity was blocked were more susceptible to disease induction in several models of experimental autoimmune disease, it remained unclear by which mechanism TRAIL suppressed the induction of autoimmunity. Disease induction in these experimental models is complex and often involves adjuvants that stimulate DCs and innate immune cell networks that may affect TRAIL function, or activation-induced T cell death in the periphery. Thus, it is important not to over-interpret findings in experimental models of autoimmune disease. Moreover, many therapies that are effective in EAE, are ineffective in MS patients (Pender and Wolfe, 2002). Furthermore, in Chapter 6, we demonstrate that aged C57BL/6 TRAIL^{-/-} mice do not develop spontaneous autoimmune disease, suggesting that

TRAIL function is not critical for the suppression of spontaneous autoimmunity in these mice.

Recently, TRAIL has been identified as a potential response marker for IFN- β therapy in MS patients (Wandinger et al., 2003). Patients with raised levels of soluble TRAIL in their sera prior to IFN- β therapy and those who showed early and sustained TRAIL induction after therapy were most likely to respond to treatment (Wandinger et al., 2003). PBLs isolated from MS patients have also been reported to express higher levels of TRAIL than healthy control subjects (Huang et al., 2000), but it has yet to be investigated whether levels of PBL TRAIL expression correlate with response to IFN- β therapy. Although it is not yet determined whether TRAIL can regulate MS, our study has suggested that administration of rTRAIL/Apo2L or anti-DR5 mAb may prove to be an effective strategy for dampening the autoimmune response.

CHAPTER SIX

NORMAL THYMOCYTE NEGATIVE SELECTION IN TRAIL-DEFICIENT MICE

6.1 Abstract

Negative selection is essential for deletion of potentially self-reactive thymocytes and the generation of a largely self-tolerant peripheral T cell repertoire. Although a number of factors have been identified to play a role in negative selection, the signaling pathway(s) involved are not fully understood. In particular, the involvement of the DR subgroup of the TNF superfamily has been the subject of many investigations, with equivocal results. A recent report suggested that TRAIL-deficient mice have defective negative selection of thymocytes. By contrast, previous studies had indicated that inhibition of the FADD-caspase 8 pathway necessary for TRAIL signaling, or direct inhibition of TRAIL using inhibitors, each failed to prevent negative selection of T cells. In Chapter 5, TRAIL was demonstrated to suppress autoimmune damage in three different models of EAE. Since impaired negative selection of T cells might explain a predisposition of TRAIL-deficient mice to development of autoimmune disease, we sought to investigate intrathymic negative selection in these mice. Using several well-established models including: antibody-mediated TCR/CD3 ligation *in vitro* and *in vivo*; stimulation with endogenous superantigen *in vitro* and *in vivo*; stimulation of TCR transgenic thymocytes with agonist peptide *in vitro*; and treatment with exogenous superantigen *in vitro*, we were unable to demonstrate a critical role for TRAIL signaling in negative selection of thymocytes. Moreover, we failed to reveal any signs of spontaneous autoimmunity in aged C57BL/6 TRAIL^{-/-} or BALB/c TRAIL^{-/-} mice, suggesting a maintenance of immunological tolerance in mice deficient in TRAIL.

6.2 Introduction

Within the thymus, BM-derived stem cells undergo a series of maturation events and prior to export, undergo thymic selection to ensure that the majority of thymocytes entering the periphery are capable of interacting with self-MHC with low affinity. DP thymocytes that express TCRs with a low affinity for peptide-MHC undergo positive selection (Anderson et al., 1993) and can exit to the periphery where they circulate throughout the secondary lymphoid organs awaiting encounter with antigen (Goldrath and Bevan, 1999). By contrast, DP thymocytes that express TCRs that recognize self peptide-MHC with a high affinity and are potentially self-reactive, undergo negative selection leading to apoptosis (Palmer, 2003). Negative selection is essential for the generation of a self-tolerant peripheral T cell repertoire and recent studies have reiterated the importance of negative selection in the prevention of autoimmune disease (Anderson et al., 2002; Bouillet et al., 1999; Bouillet et al., 2002; Liston et al., 2003). Depending on the model system used, negative selection appears to occur at the immature DP stage of thymocyte differentiation in the thymic cortex and possibly later at the SP HSA^{hi} semi-mature stage when thymocytes transit through the CMJ (Stockinger, 1999).

The molecular mechanisms which underly the death of autoreactive thymocytes in negative selection are yet to be fully understood, although a number of factors have been identified to play a role in the process. A number of intracellular signaling factors have been shown to be important for negative selection, including the Nur-77/Nurr1/Nor1 transcription factor family (Calnan et al., 1995; Cheng et al., 1997; Lee et al., 1995) and the pro-apoptotic Bcl-2 family members Bim (Bouillet et al., 2002), Bax and Bak (Rathmell et al., 2002). More controversial is the contribution made by the DRs; members of the tumor necrosis factor receptor family containing an intra-cellular DD (eg. Fas, TNF-R1 and TRAIL receptors) (DeYoung et al., 2000; Page et al., 1998; Sprent and Kishimoto, 2002; Wang et al., 2001a; Wang et al., 2001b).

Several members of the TNF superfamily have been investigated for their role in negative selection, including: TRAIL, Fas, TNF, DR3, LT, LIGHT and CD30 (Amakawa et al., 1996; Chiarle et al., 1999; DeYoung et al., 2000; Grech et al., 2000; Kishimoto and Sprent, 1999; Kishimoto et al., 1998; Lamhamedi-Cherradi et al., 2003; Page, 1999; Page et al., 1998; Simon et al., 2001; Sprent and Kishimoto, 2002; Wang et al., 2001a; Wang et al., 2001b). These studies have yielded conflicting results, making it unclear whether the TNF superfamily plays a role in the process. For example, Kishimoto and colleagues suggest a role for Fas at high, but not low intensity TCR stimulation (Kishimoto et al., 1998), whereas other investigators propose that Fas signaling is dispensable for negative selection (Sidman et al., 1992; Villunger et al., 2004). Importantly, negative selection occurs normally in FADD dominant-negative mice (Newton et al., 1998) and in transgenic mice lacking caspase-8 function (Smith et al., 1996), questioning the involvement of the DRs (that utilize this pathway to induce apoptosis) in negative selection.

Recently, a critical role for TRAIL in negative selection was proposed by Lamhamedi-Cherradi and colleagues (Lamhamedi-Cherradi et al., 2003). Negative selection was demonstrated to be at least partially impaired in TRAIL-deficient mice or in the presence of blocking soluble TRAIL receptor (DR5), using several *in vitro* and *in vivo* models, including: antibody-mediated TCR/CD3 ligation *in vitro* and *in vivo*, stimulation with endogenous superantigen *in vivo*, and stimulation with antigenic peptide *in vivo*. This report clearly contrasted with a previous study that used soluble blocking TRAIL-R2-Fc to demonstrate that negative selection of mouse and human thymocytes was TRAIL-independent (Simon et al., 2001), and studies that excluded a role for the FADD-caspase 8 pathway (Newton et al., 1998; Smith et al., 1996). To explain these discrepancies, it has been proposed that TRAIL might utilize an alternative (as yet undefined) apoptosis signaling pathway that does not involve FADD-caspase 8, but may activate Bim and Bax/Bak (Green, 2003).

In this study, we sought to investigate the role of TRAIL in thymocyte negative selection using TRAIL-deficient mice and a powerful neutralizing anti-mTRAIL

mAb. We demonstrate that TRAIL does not play a role in thymocyte negative selection using several well-established models. These include: antibody-mediated TCR/CD3 ligation *in vitro* and *in vivo*; deletion of endogenous superantigen reactive T cells *in vivo* and *in vitro*; stimulation with the superantigen SEB *in vitro*; and *in vitro* stimulation of TCR transgenic thymocytes with agonist peptide. Development of spontaneous autoimmune disease in aged C57BL/6 TRAIL^{-/-} mice was also monitored.

6.3 Materials and Methods

Mice

Inbred BALB/c WT, C57BL/6 WT and C57BL/6 OT-I TCR transgenic mice were purchased from The WEHI. BALB/c TRAIL^{-/-}, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-} *gld* mice were obtained and bred at Peter Mac as detailed in Chapter 2, Section 2.3 and Chapter 4, Section 4.3, Materials and Methods. To obtain timed pregnancies, mice were mated for 15 h and the embryos removed at fetal day (FD) 15 or 18 (plug date = day 0). Age and sex matched mice, 5-18 weeks of age were used in all experiments (unless otherwise stated), under specific pathogen-free conditions according to Animal Experimental Ethics Committee guidelines and approval.

FTOC

Fetal thymic lobes were isolated from day 15 BALB/c WT and BALB/c TRAIL^{-/-} embryos and cultured for 12 days in RPMI-1640 medium supplemented with 10% FCS, 2 mM GlutaMAX, 100 IU/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES buffer (Invitrogen Life Technologies Corp), and 1 mM sodium pyruvate (Invitrogen Life Technologies Corp) with or without 20 µg/ml anti-mTRAIL mAb (N2B2) (prepared as detailed in Chapter 2, Section 2.1, Materials and Methods) and 10 µg/ml SEB (Toxin Technology Co., Sarasota, FL, USA). Lobes were cultured in groups of 2 per well of a 6-well plate and medium was changed after 6 days. At the end of culture, thymocytes were harvested from lobes, counted and analyzed by flow cytometry.

Fetal thymic lobes were also isolated from day 15 C57BL/6 OT-I TCR transgenic embryos and cultured for 7 days in supplemented media with or without 200 µg/ml anti-mTRAIL mAb and 1-10,000 nM OVA₂₅₇₋₂₆₄ peptide (SIINFEKL). In other experiments, fetal thymic lobes were isolated from day 15 C57BL/6 OT-I TCR transgenic embryos, cultured for 3 days in supplemented media and then cultured for 30 min. in the presence or absence of 200 µg/ml anti-mTRAIL mAb. OVA₂₅₇₋₂₆₄ peptide (20 µM) was then added to select wells and the cultures incubated a further 24 h to induce deletion. Lobes were cultured in groups of 2

per well of a 6-well plate. At the end of culture, thymocytes were harvested from lobes, counted and analyzed by flow cytometry.

Anti-CD3 ligation *in vitro*

For *in vitro* analysis, thymocyte suspensions were produced from thymic lobes isolated from day 18 BALB/c WT and BALB/c TRAIL^{-/-} embryos or from thymi isolated from adult BALB/c WT, BALB/c TRAIL^{-/-}, C57BL/6 WT, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* or C57BL/6 TRAIL^{-/-} *gld* mice. Cells were cultured at a density of 1×10^6 cells/ml, in DMEM medium supplemented with 10% FCS in wells of a 96-well plate that had been coated overnight or at 37°C for 1 h with hamster anti-mouse CD3 ϵ (145-2C11), anti-CD28 (37.51) mAb (both sourced from BD Biosciences Pharmingen) and/or PBS. Twenty hours later, proportions of live and apoptotic cells were identified by staining with propidium iodide (PI) (Sigma Chemical Co) and annexin V-FITC (Roche, IN, USA). Live cells were considered to be PI⁻ and annexin V- FITC⁻.

Anti-CD3 antibody treatment *in vivo*

Eight week old BALB/c and BALB/c TRAIL^{-/-} mice were injected i.p. with either 20 μ g anti-CD3 mAb (145-2C11), or vehicle control, PBS. Forty hours after injection, mice were sacrificed, their thymi and spleens isolated, cells counted and analyzed by flow cytometry.

Thymic stromal cell isolation

This procedure was adapted by Dr. Daniel Gray (Monash Medical School, Prahran, Victoria, Australia) (Gray et al., 2002) from (Shortman et al., 1989). Thymi were dissected from 5-7 week old C57BL/6 WT mice and trimmed of fat and connective tissue. Small cuts into the capsules were made with a pair of fine scissors and the lobes were gently agitated in 50 ml of RPMI-1640 with a magnetic stirrer at 4°C for 30 min. to remove the majority of thymocytes. The resulting thymic fragments were transferred into 10 ml of fresh RPMI-1640 and dispersed further with a wide bore glass pipette to free more thymocytes. The thymic fragments were then incubated in 5 ml of 0.125% (w/v) collagenase D and 0.1% (w/v) DNase I (both obtained from Boehringer Mannheim, Penzberg,

Germany) in RPMI-1640 at 37°C for 15 min., with gentle agitation using a Pasteur pipette every 5 min. Enzyme mixtures with isolated cells were removed after fragments had settled, then replaced with fresh mixture for further incubation. Gentle mechanical agitation was performed with a 200 µl pipette tip to break up aggregates remaining in final digestions. After 4-5 digestions, cells from the final 1-2 digests were pooled and centrifuged at 450 x g for 5 min., and resuspended in 5 mM EDTA in FACS buffer. Thymic stromal cells were then passed through 100 µm mesh to remove debris prior to cell counts. Thymic epithelial cells were identified by flow cytometry as CD45⁺EpCAM⁺ and thymic DCs as CD11c⁺MHC-II^{hi}.

Annexin-V / PI staining

Cells were washed three times in annexin-V binding buffer (0.01 M Hepes, 0.14 M NaCl, 2.5 mM CaCl₂), before labelling with FITC-conjugated Annexin-V for 25 min. at RT, protected from light. Cells were washed once and resuspended in annexin-V buffer. PI was added to a final concentration of 0.5 µg/ml and the cells immediately analyzed by flow cytometry.

Flow cytometric analysis

Thymocytes, splenocytes, LN, epithelial and DCs were stained with surface marker-specific mAbs including: anti-TCRβ-APC (H57-597), anti-CD3-FITC (17A2), anti-CD4-PerCP-Cy5.5, -FITC and -APC (RM4-5), anti-CD8α-PE, -APC, -PerCP and -FITC (53-6.7), anti-CD25-PE (PC61), anti-CD69-PE (H1.2F3), anti-HSA (CD24)-PE (M1/69), anti-IA/IE-FITC and -PE (M5/114.15.2), anti-EpCam (G8.8), anti-mTRAIL-PE (N2B2), anti-CD45-APC (30-F11), anti-CD11b-APC (M1/70), anti-DR5-biotin (MD5-1), anti-CD11c-FITC and -biotin (HL3), anti-Vα2-PE (B20.1), anti-TCRVβ3-biotin (KJ25), anti-TCRVβ5.1/5.2-biotin (MR9-4), anti-TCRVβ6-biotin (RR4-7), anti-TCRVβ8.1-8.2-biotin (MR5-2) and anti-Vβ11-biotin (RR3-15) in the presence of anti-Fcγ receptor block CD16/32 (2.4G2). Biotinylated antibodies were detected with streptavidin-APC or streptavidin-CyChrome (BD Biosciences Pharmingen) and anti-EpCam was detected with polyclonal anti-rat-FITC (Silenus Laboratories, Hawthorn, Victoria, Australia). Anti DR5-biotin was kindly provided by Dr.

Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan), anti-EpCam was kindly provided by Dr. Andy Farr (Department of Biological Structure, University of Washington, Seattle, WA, USA), anti-mTRAIL-PE was obtained from e-Bioscience and all remaining antibodies from BD Biosciences Pharmingen. Analysis was performed on a FACSCalibur™ and analyzed using the CELLQuest™ software.

Histology

For aging experiments, C57BL/6 and C57BL/6 TRAIL^{-/-} mice were sacrificed at > 555 days and a full autopsy performed. Kidney, spleen and pancreas were examined for signs of autoimmune disease (inflammation and tissue destruction) by histology (H&E staining) after fixing these tissues in formalin. The preparation and staining of sections for histology was carried out by Brenda Aisbett (Histology Laboratory, Peter Mac).

Serum ELISAs

Sera from aged C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice and from 28 week C57BL/6 *gld* mice (as a positive control) was tested by ELISA for total IgG and anti-dsDNA antibodies as detailed in Chapter 4, Section 4.3, Materials and Methods.

Statistical analysis

Data were analyzed using a two tailed, non-parametric, unpaired Mann Whitney test. P values < 0.05 were considered significant.

6.4 Results

6.4.1 Anti-CD3/anti-CD28 mAb-induced apoptosis *in vitro*

One widely used model of negative selection is *in vitro* exposure of thymocytes to anti-CD3 mAb, which aggregates the TCR-CD3 complex and induces apoptosis. Thymic lobes isolated from E18 mice are a rich source of DP immature thymocytes (90-95%) and importantly lack mature T cells that upon TCR/CD3 ligation might release soluble factors that kill DP thymocytes indirectly. *In vitro* stimulation with anti-CD3 mAb induced apoptosis of E18 BALB/c WT thymocytes in a dose-dependent manner as determined by staining with FITC-coupled annexin V, which detects externalized phosphatidyl-serine, a hallmark of apoptotic cells (Figure 6.1). Stimulation with anti-CD3 plus anti-CD28 mAb induced higher levels of thymocyte apoptosis compared to stimulation with anti-CD3 mAb alone (Figure 6.1). Equivalent levels of apoptosis were induced in BALB/c WT, BALB/c TRAIL^{-/-} and BALB/c WT thymocytes incubated with a neutralizing anti-mTRAIL mAb at all concentrations of anti-CD3 and anti-CD28 mAb tested (Figure 6.1). The spontaneous death of BALB/c WT, BALB/c TRAIL^{-/-} and BALB/c WT thymocytes incubated with anti-mTRAIL mAb in culture was also equivalent (Figure 6.1).

We then examined *in vitro* the anti-CD3 plus anti-CD28 mAb-induced apoptosis of thymocytes isolated from adult mice. Apoptosis of total (Figure 6.2A) and DP (Figure 6.2B) thymocytes was examined by staining with FITC-coupled annexin V. Similar results were obtained for total thymocytes and DP thymocytes that are known to be susceptible to negative selection. Stimulation with anti-CD3 mAb, with or without anti-CD28 mAb induced equivalent apoptosis in BALB/c WT and BALB/c TRAIL^{-/-} thymocytes and in C57BL/6 WT and C57BL/6 TRAIL^{-/-} thymocytes (Figure 6.2A and B). Stimulation with anti-CD3 plus anti-CD28 mAb induced higher levels of total thymocyte apoptosis compared to stimulation with anti-CD3 mAb alone (Figure 6.2A and B). The spontaneous death of BALB/c WT and BALB/c TRAIL^{-/-} thymocytes and of C57BL/6 WT and C57BL/6 TRAIL^{-/-} thymocytes in culture was also equivalent (Figure 6.2A and B). Interestingly, the spontaneous death of thymocytes isolated from mice of

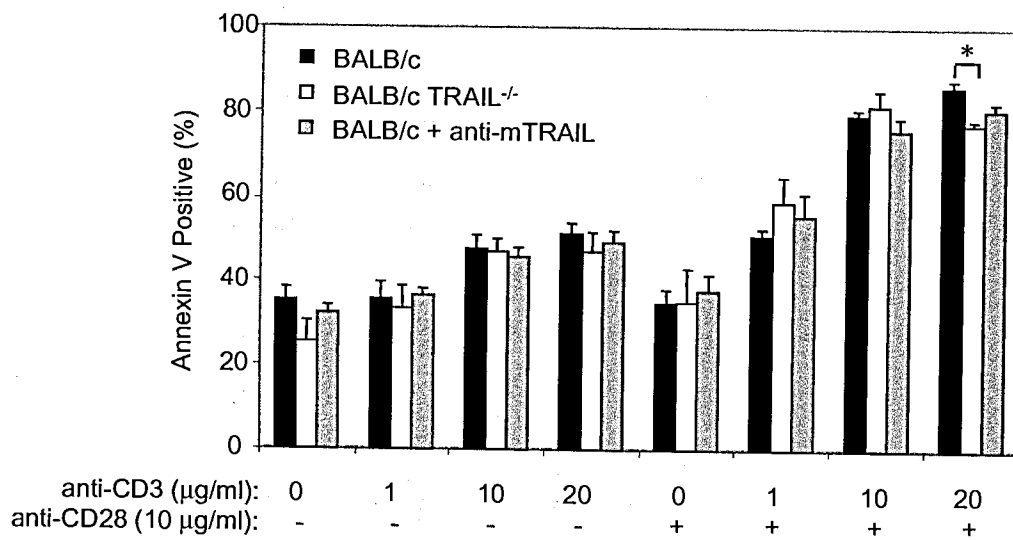


Figure 6.1. Anti-CD3 plus anti-CD28 mAb-induced apoptosis of day 18 embryo (E18) thymocytes *in vitro*. Thymocytes from BALB/c WT and BALB/c TRAIL^{-/-} E18 mice were cultured in plates coated with anti-CD3 ϵ (145-2C11) plus anti-CD28 (37.51) mAbs and/or PBS. Neutralizing anti-mTRAIL mAb was added to some of the cultures. After 20 h, live and apoptotic cells were discriminated by staining with annexin V-FITC and flow cytometric analysis. Data represent mean \pm SEM, for thymocytes isolated from 6 BALB/c mice with or without the addition of anti-mTRAIL mAb, or 4 BALB/c TRAIL^{-/-} mice. Asterisk indicates groups that are significantly different (* $P < 0.05$).

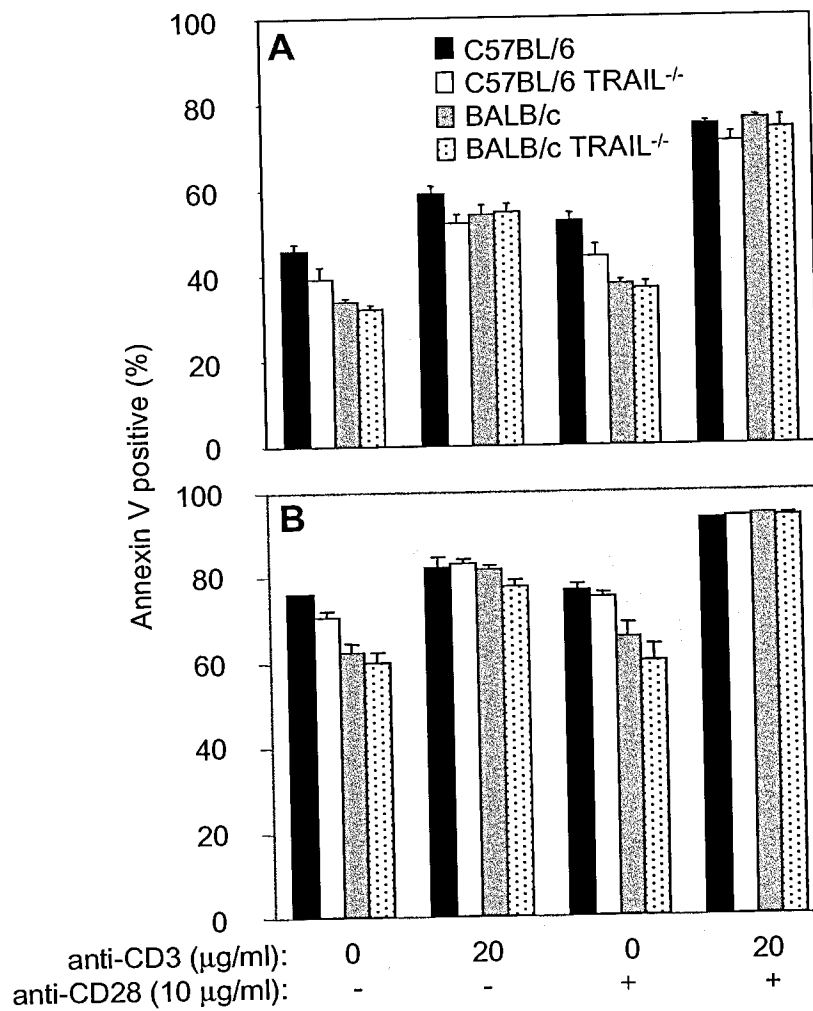


Figure 6.2. Anti-CD3 plus anti-CD28 mAb-induced apoptosis of adult thymocytes *in vitro*. Thymocytes isolated from adult BALB/c, BALB/c TRAIL^{-/-}, C57BL/6 and C57BL/6 TRAIL^{-/-} mice were cultured in plates coated with anti-CD3ε (145-2C11) plus anti-CD28 (37.51) mAbs and/or PBS. After 20 h, live and apoptotic cells were discriminated by staining with annexin V-FITC and flow cytometric analysis. Percentage annexin V⁺ thymocytes is illustrated for (A) total thymocytes and (B) CD4⁺CD8⁺ DP thymocytes. Data represent mean ± SEM for 3 BALB/c, BALB/c TRAIL^{-/-}, C57BL/6 and C57BL/6 TRAIL^{-/-} mice. Percentage annexin V⁺ is not significantly different for C57BL/6 and C57BL/6 TRAIL^{-/-} ($P > 0.05$) or BALB/c and BALB/c TRAIL^{-/-} mice ($P > 0.05$) for any of the stimuli shown.

C57BL/6 background was significantly higher than that observed for thymocytes isolated from mice of BALB/c background (Figure 6.2A and B), suggesting that C57BL/6 WT thymocytes were more sensitive to the overnight culture conditions than BALB/c WT thymocytes.

A role for Fas in negative selection remains controversial. Although one report suggests that Fas plays a role in negative selection against high, but not low affinity ligands (Kishimoto et al., 1998) other reports suggest that Fas signaling is dispensable for negative selection (Sidman et al., 1992; Villunger et al., 2004). To evaluate the potential contribution of FasL and determine whether redundancy operated between the FasL and TRAIL signaling pathways, negative selection was compared in adult C57BL/6 *gld* (FasL mutant) and C57BL/6 TRAIL^{-/-}*gld* thymocytes. Apoptosis of total (Figure 6.3A) and DP (Figure 6.3B) thymocytes was examined by staining with FITC-coupled annexin V. Similar results were obtained for total thymocytes and DP thymocytes. Stimulation with anti-CD3 mAb, with or without anti-CD28 mAb induced equivalent apoptosis in C57BL/6 WT, C57BL/6 *gld*, C57BL/6 TRAIL^{-/-} and C57BL/6 TRAIL^{-/-}*gld* thymocytes (Figure 6.3A and B). Stimulation with anti-CD3 plus anti-CD28 mAb induced higher levels of thymocyte apoptosis compared to stimulation with anti-CD3 mAb alone (Figure 6.3A and B). The spontaneous death of C57BL/6 WT, C57BL/6 *gld*, C57BL/6 TRAIL^{-/-} and C57BL/6 TRAIL^{-/-}*gld* thymocytes in culture was also equivalent (Figure 6.3A and B). Together, these data suggest that neither TRAIL, nor FasL plays a role in negative selection of thymocytes. Furthermore, TRAIL and FasL do not function cooperatively to induce negative selection *in vitro*.

6.4.2 SIINFEKL-induced depletion of OT-I thymocytes developing in fetal thymic organ culture (FTOC)

Our next study examined intrathymic deletion of C57BL/6 OT-I TCR transgenic thymocytes induced by agonist peptide in FTOC. Acute deletion of DP thymocytes was measured by culturing E15 C57BL/6 OT-I thymic lobes for 3 days to allow the development of DP thymocytes and then stimulating for 24 h in the presence or absence of SIINFEKL peptide (OVAp) and neutralizing anti-

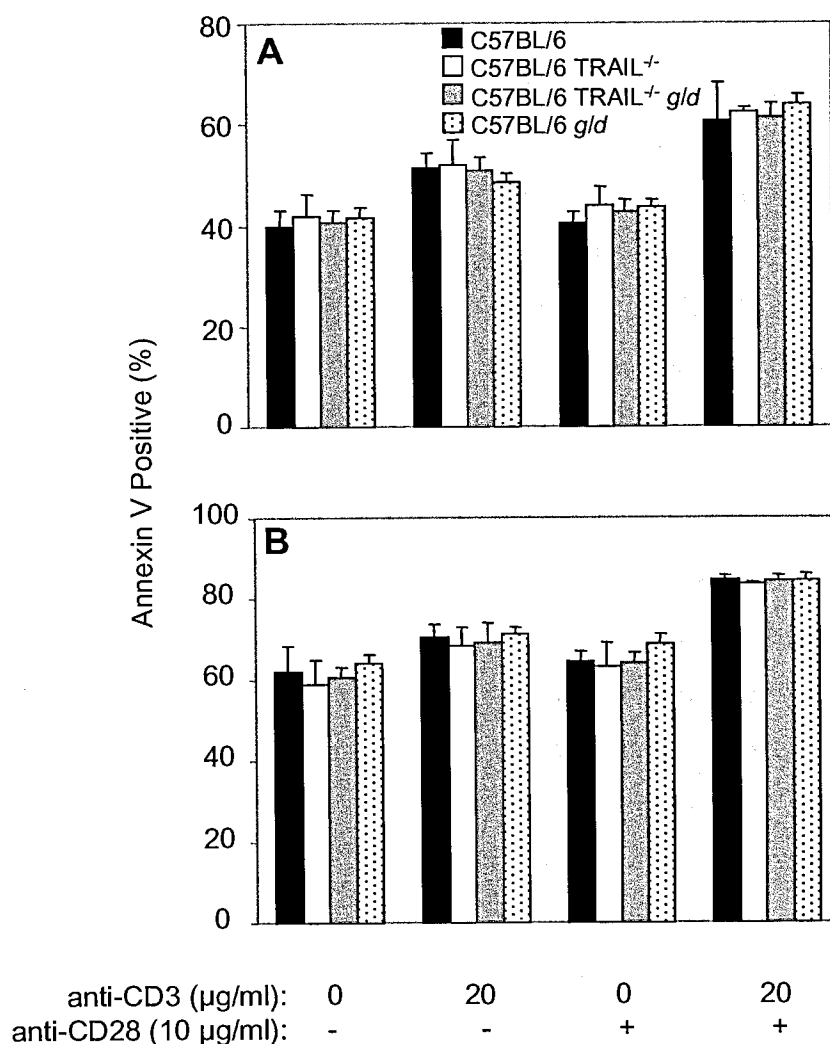


Figure 6.3. Anti-CD3 plus anti-CD28 mAb-induced apoptosis of adult C57BL/6 *gld* and C57BL/6 TRAIL^{-/-}*gld* thymocytes *in vitro*. Thymocytes isolated from adult C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 TRAIL^{-/-}*gld* and C57BL/6 *gld* mice were cultured in plates coated with anti-CD3ε (145-2C11) plus anti-CD28 (37.51) mAbs and/or PBS. After 20 h, live and apoptotic cells were discriminated by staining with annexin V-FITC and flow cytometric analysis. Percentage annexin V⁺ thymocytes is illustrated for (A) total thymocytes and (B) CD4⁺CD8⁺ DP thymocytes. Data represent mean ± SEM for 3 C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 *gld* and C57BL/6 TRAIL^{-/-}*gld* mice. Percentage annexin V⁺ was not significantly different for C57BL/6, C57BL/6 TRAIL^{-/-}, C57BL/6 TRAIL^{-/-}*gld* and C57BL/6 *gld* mice for any of the stimuli shown ($P > 0.05$).

mTRAIL mAb. OVA_p induced complete deletion of OT-I DP thymocytes, and the addition of neutralizing anti-mTRAIL mAb afforded no protection against negative selection (Figure 6.4A). A proportion of SP thymocytes were also deleted in a TRAIL-independent manner upon addition of OVA_p (Figure 6.4A). Deletion of DP and SP thymocytes in cultures incubated with agonist peptide was reflected by a decrease in total cell numbers per thymus lobe (Figure 6.4B) and an increase in the percentage of cells undergoing apoptosis as determined by staining with FITC-coupled annexin-V (Figure 6.4C).

Although TRAIL was not required for acute deletion of C57BL/6 OT-I thymocytes stimulated with agonist peptide, the question remained whether TRAIL might play a role in negative selection at lower levels of TCR signaling. To help answer this question, a long-term culture of E15 C57BL/6 OT-I thymic lobes was performed [as in (Hogquist et al., 1994)] in 0 to 10⁻⁵M OVA_p in the presence or absence of anti-mTRAIL mAb for a period of 7 days. Stimulation with OVA_p deleted both DP and SP thymocytes in a dose-dependent manner and addition of neutralizing anti-mTRAIL mAb had no effect on deletion (Figure 6.5). These results suggest that TRAIL does not play a critical role in thymocyte negative selection induced by agonist peptide *in vitro*.

6.4.3 *Staphylococcus enterotoxin B* (SEB)-induced deletion of V β 8⁺ T cells developing in FTOC

Stimulation with the superantigen SEB activates and deletes the majority of T cells that express a variable region (V) β 8 TCR, whereas T cells that express TCR V β 6 do not respond to SEB and remain unaffected (Jenkinson et al., 1990). The effect of TRAIL-deficiency on SEB-induced deletion of TCRV β 8 cells was examined in FTOC. Equivalent SEB-induced deletion of TCRV β 8⁺ T cells was observed in BALB/c WT, BALB/c TRAIL^{-/-} and BALB/c WT fetal thymus lobes incubated with a neutralizing anti-mTRAIL mAb (Figure 6.6). SEB promoted deletion of both CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ subsets of TCRV β 8⁺ T cells, whilst TCRV β 6⁺ T cells that do not respond to SEB were unaffected (Figure 6.6). These data further substantiated the argument that TRAIL is not required for negative selection of thymocytes.

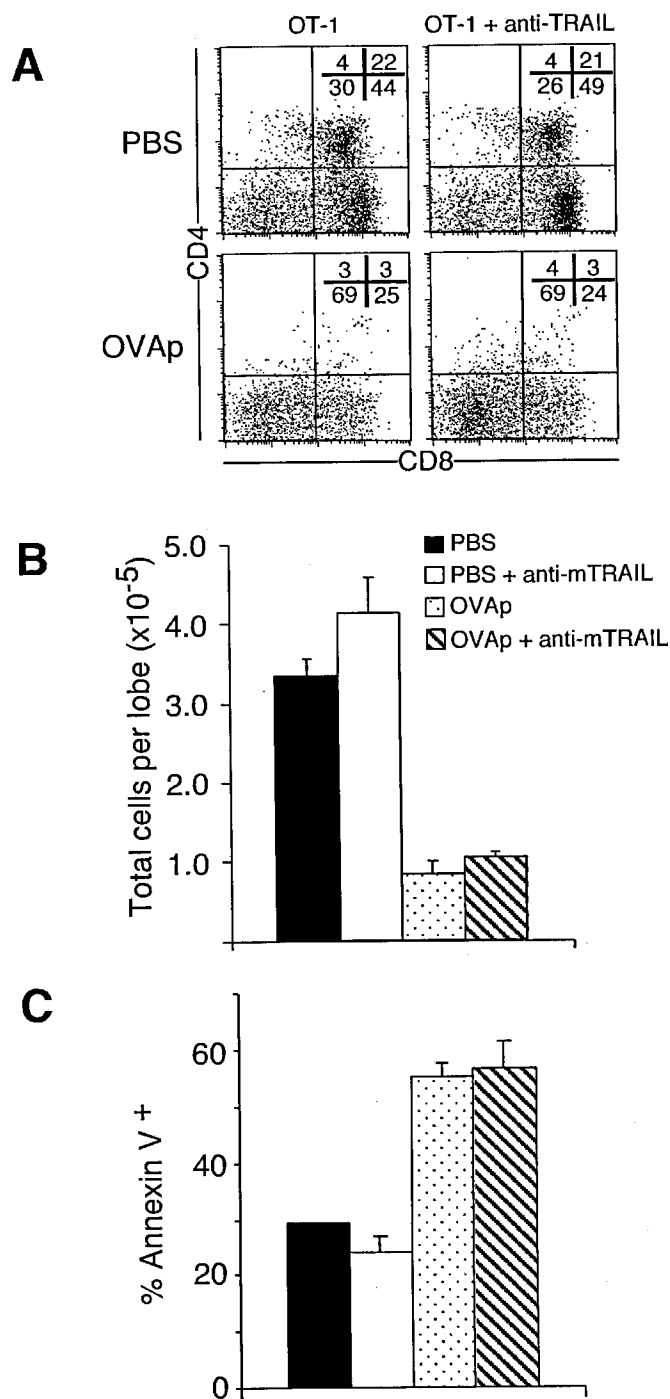


Figure 6.4. SIINFEKL-induced depletion of OT-I thymocytes developing in FTOC. Thymic lobes isolated from E15 OT-I TCR transgenic mice were cultured for 3 days to allow development of DP thymocytes. Acute deletion was induced by the addition of 20 μ M agonist peptide (OVAp, amino acid sequence SIINFEKL) or an equivalent volume of PBS as negative control, in the presence or absence of 200 μ g/mL anti-mTRAIL mAb. Twenty-four hours after the addition of OVAp, thymocytes were harvested, cells counted and stained with antibodies to CD4, CD8, CD3 or V α 2. (A) Representative CD4 versus CD8 FACS profiles (B) total cells per lobe and (C) % annexin V⁺ are shown. Mean \pm SEM were calculated from analysis of 2 samples per group, each containing 2 thymic lobes. Total cells per lobe and percentage annexin V⁺ are not significantly different for lobes incubated with or without anti-mTRAIL mAb.

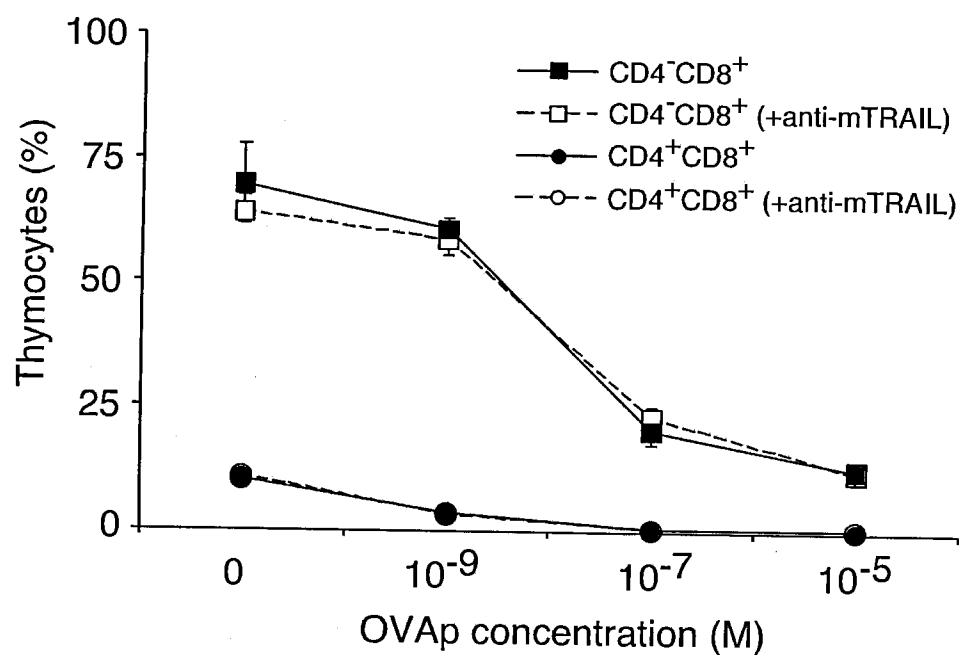


Figure 6.5. SIINFEKL-induced depletion of OT-I thymocytes at lower TCR avidities. Thymic lobes isolated from E15 OT-I TCR transgenic mice were cultured for 7 days with 0 to 10 μ M OVAp in the presence or absence of 200 μ g/ml anti-mTRAIL mAb or the equivalent volume of PBS. Percentage CD8-SP and DP thymocytes, in the presence or absence of anti-mTRAIL mAb, is shown at various peptide concentrations. Mean \pm SEM were calculated from analysis of 3-4 samples per group, each containing 2 thymic lobes. Percentage CD8-SP and DP thymocytes are not significantly different for lobes incubated with or without anti-mTRAIL mAb.

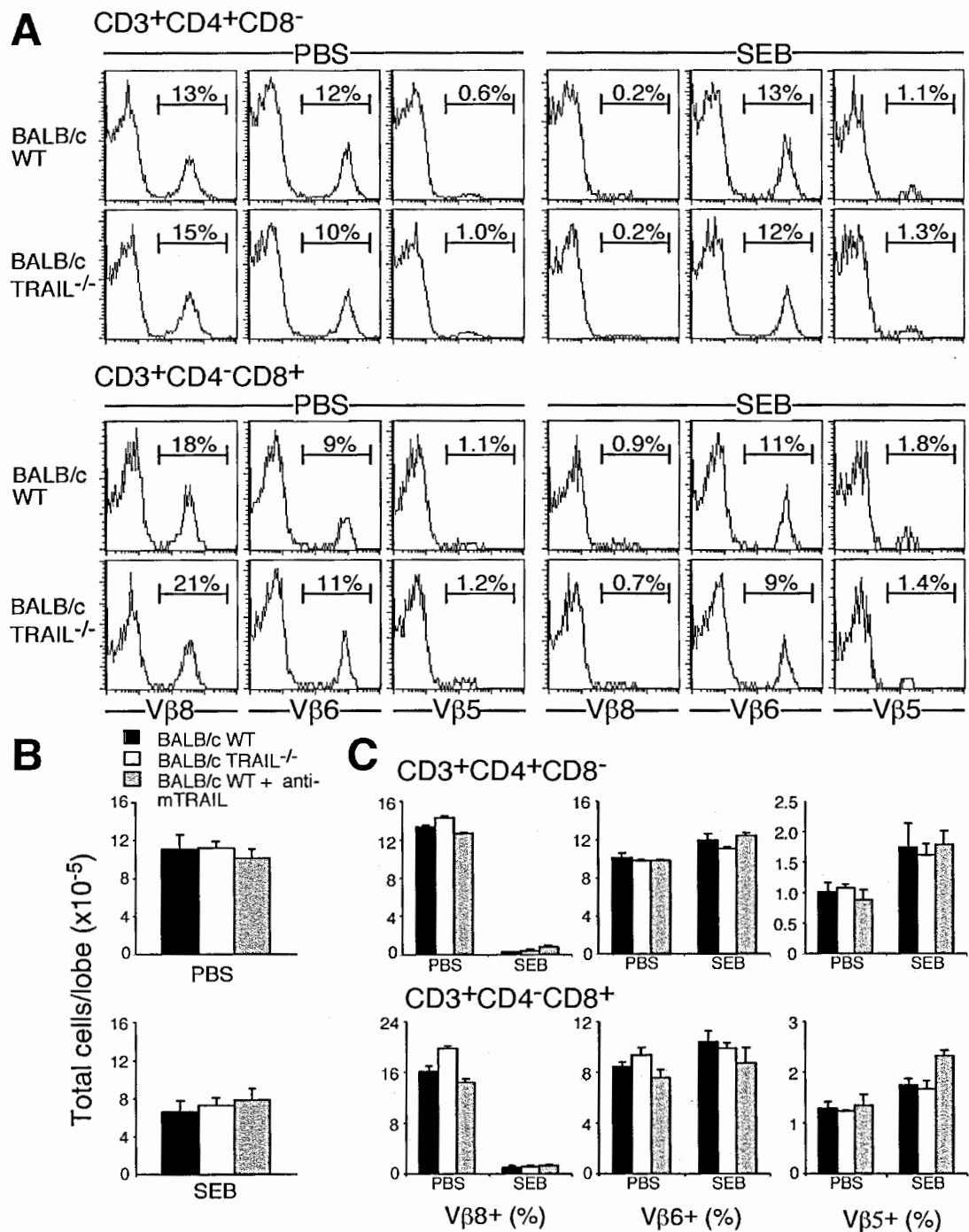


Figure 6.6. SEB-induced deletion of TCR Vβ8⁺ T cells developing in FTOC. Thymic lobes isolated from E15 BALB/c WT and BALB/c TRAIL^{-/-} mice were cultured for 12 days in the presence or absence of 10 μg/ml SEB plus or minus 20 μg/ml anti-mTRAIL mAb or an equivalent volume of PBS. Thymocyte suspensions were isolated, counted and stained with antibodies to CD4, CD8, CD3 and TCR Vβ8, Vβ6 or Vβ5. (A) Histograms demonstrate expression of TCR Vβ8, Vβ6 or Vβ5 on CD3^{high} thymocytes (CD4⁺ or CD8⁺) after incubation with SEB or PBS. (B) Total cells per thymic lobe. (C) TCR Vβ8⁺, TCR Vβ6⁺ or TCR Vβ5 cells as a percentage of CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ thymocyte subsets. Mean ± SEM were calculated from analysis of 6 BALB/c TRAIL^{-/-} and 8 BALB/c WT thymic lobes for both PBS and SEB treatment.

6.4.4 Mouse mammary tumor virus (MMTV)6 and MMTV9 deletion of $V\beta 3^+$ and $V\beta 5^+$ T cells

To determine whether TRAIL plays a role in negative selection induced by endogenous antigens, MMTV6 and MMTV9-induced negative selection was investigated *in vitro* and *in vivo*. MMTV6 and MMTV9 encode endogenous superantigens that selectively delete $TCRV\beta 5^+$ and $TCRV\beta 3^+$ T cells in mice that express the class II MHC antigen (I-E) (Tomonari et al., 1993; Woodland et al., 1991). To investigate the effect of endogenous superantigen *in vitro*, BALB/c (I-E⁺) $TCRV\beta 5^+$ T cells developing in FTOC were examined for deletion by MMTV6 and 9. TRAIL deficiency, or addition of neutralizing anti-mTRAIL mAb did not protect developing $TCRV\beta 5^+$ T cells from deletion (Figure 6.6A and B). Intrathymic deletion of $TCRV\beta 5^+$ and $TCRV\beta 3^+$ T cells by MMTV6 and 9 were also assessed in adult BALB/c WT mice *in vivo*. The frequencies of $TCRV\beta 5^+$ and $TCRV\beta 3^+$ cells amongst $CD4^+$ and $CD8^+$ subsets were examined in thymus, spleen and pLNs isolated from BALB/c WT, BALB/c TRAIL^{-/-} and C57BL/6 WT mice (Figure 6.7A and B). Similar deletion of $CD4^+$ and $CD8^+$ subsets of $TCRV\beta 5^+$ and $TCRV\beta 3^+$ T cells was observed in BALB/c WT and BALB/c TRAIL^{-/-} mice, whereas cells with these specificities were not deleted in C57BL/6 WT mice (I-E⁻) (Figure 6.7A and B). As a negative control, frequencies of $TCRV\beta 6^+$ and $TCRV\beta 8^+$ T cells that are not deleted by MMTV6 and 9 are shown (Figure 6.7A and B). These data indicate that TRAIL is not required for intrathymic deletion induced by endogenous antigen *in vitro* and *in vivo*.

6.4.5 Anti-CD3 mAb mediated apoptosis *in vivo*

The final model of negative selection tested was *in vivo* exposure of thymocytes to anti-CD3 mAb. Although this model is frequently used to investigate negative selection, apoptosis induced in this manner is known to be largely TCR-independent, and is typically mediated by inflammatory cytokines and steroid hormones produced by peripheral T cells (Brewer et al., 2002; Martin and Bevan, 1997). Nevertheless, given that previous researchers had utilized this model and demonstrated a role for TRAIL in negative selection (Lamhamedi-Cherradi et al.,

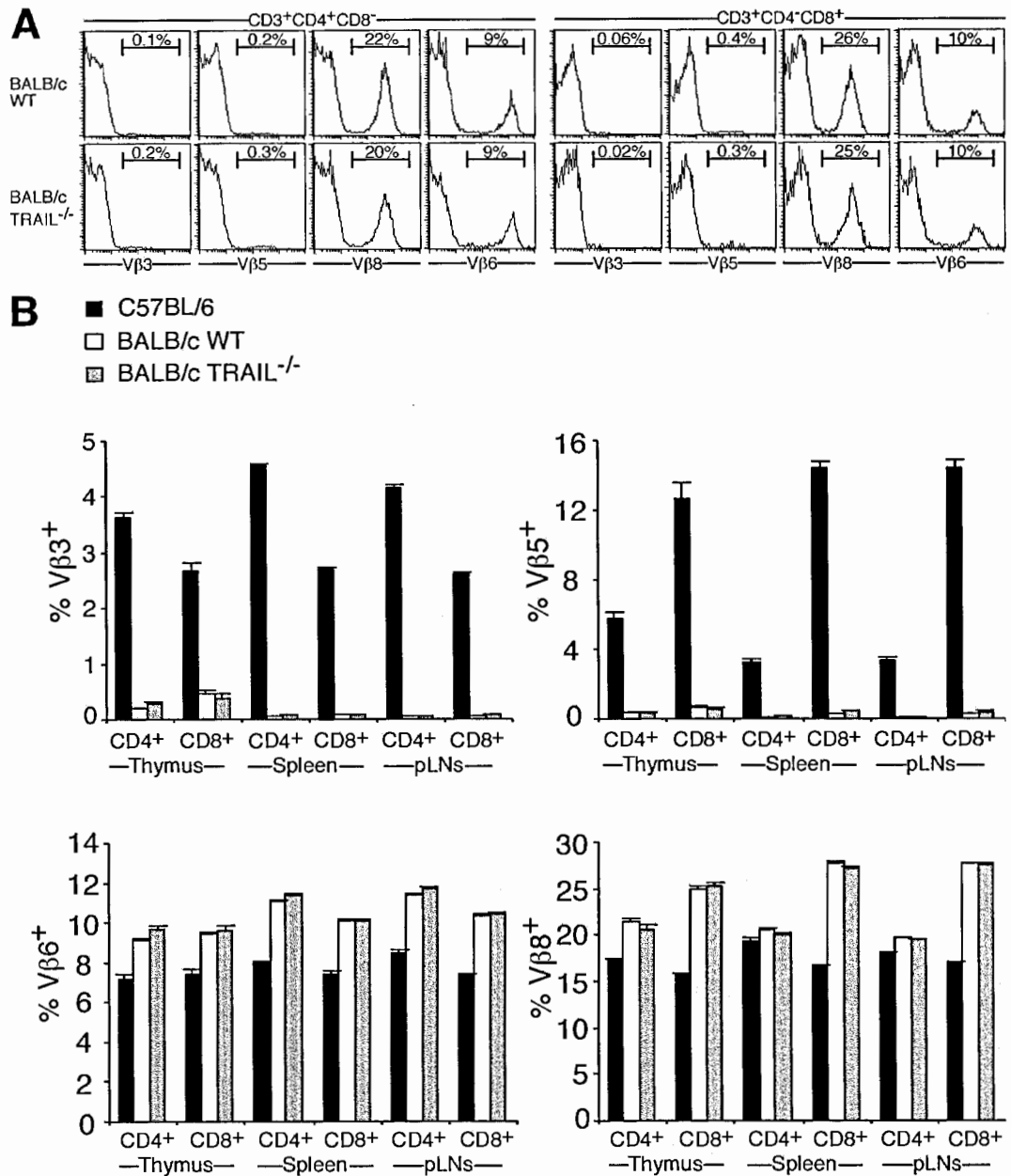


Figure 6.7. MMTV-6 and MMTV-9 induced deletion of TCR Vβ3 and TCR Vβ5 T cells *in vivo*. Thymocytes, splenocytes and pLN cells were isolated from 8 week-old male C57BL/6, BALB/c and BALB/c TRAIL^{-/-} mice. Cell suspensions were stained with antibodies to CD4, CD8, CD3 and TCR Vβ3, Vβ5, Vβ6, or Vβ8. (A) Histograms demonstrate expression of TCR Vβ3, TCR Vβ5, TCR Vβ6, or TCR Vβ8 on CD4⁺ CD3^{high} or CD8⁺ CD3^{high} thymocytes. (B) TCR Vβ3, TCR Vβ5, TCR Vβ6, or TCR Vβ8 cells as a percentage of CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ subsets of thymocytes, splenocytes and pLN cells. Mean ± SEM were calculated from analysis of cells isolated from 3 C57BL/6, 12 BALB/c and 11 BALB/c TRAIL^{-/-} mice. Data shown is pooled from 4 independent experiments.

2003), we also chose to perform such experiments. I.p. injection of anti-CD3 mAb into BALB/c WT mice depleted approximately 70-80% of DP thymocytes and equivalent deletion was observed in BALB/c TRAIL^{-/-} mice (Figure 6.8A). Depletion of DP thymocytes in mice injected with anti-CD3 mAb was reflected by a decrease in total numbers of cells per thymus and total numbers of DP cells per thymus (Figure 6.8B). Anti-CD3 mAb treatment also induced depletion of CD4⁺HSA^{hi} and CD8⁺HSA^{hi} semi-mature thymocytes, but not mature CD4⁺HSA^{lo} or CD8⁺HSA^{lo} thymocytes that have previously been shown to be resistant to negative selection (Figure 6.8C) (Kishimoto and Sprent, 1997).

As a large component of the anti-CD3 mAb-induced thymocyte death observed *in vivo* is due to inflammatory cytokines and steroid hormones produced by activated peripheral T cells, we investigated peripheral T cell activation in the spleen of mice injected with PBS or anti-CD3 mAb. Differences in peripheral activation between BALB/c WT and BALB/c TRAIL^{-/-} mice would preclude comparison of anti-CD3 mAb-induced thymocyte death in these experiments. Nonetheless, equivalent peripheral T cell activation, indicated by staining with the early activation marker CD69, was observed in BALB/c WT and BALB/c TRAIL^{-/-} mice injected with PBS or anti-CD3 mAb (Figure 6.9).

Consistent with an earlier report (Lamhamedi-Cherradi et al., 2003), the thymus of adult BALB/c TRAIL^{-/-} mice injected with PBS ($1.40 \pm 0.15 \times 10^8$ cells), was slightly enlarged compared with PBS injected BALB/c WT mice ($0.95 \pm 0.17 \times 10^8$) ($P < 0.05$, Figure 6.8B). There was no significant difference in spleen or LN cellularity between the strains. The basis for this subtle difference in thymus size is unclear, but does not appear to be due to impaired negative selection.

6.4.6 Expression of TRAIL and TRAIL receptor (DR5)

Given that the most potent inducers of negative selection in the thymus are thymic DCs and epithelial cells (Hoffmann et al., 1995; Sprent and Webb, 1995; Tanaka et al., 1993), and that target cell expression of TRAIL receptor (DR5) is required for sensitivity to TRAIL-mediated apoptosis, we next examined TRAIL and DR5 expression on various cell types within the thymus. If TRAIL was

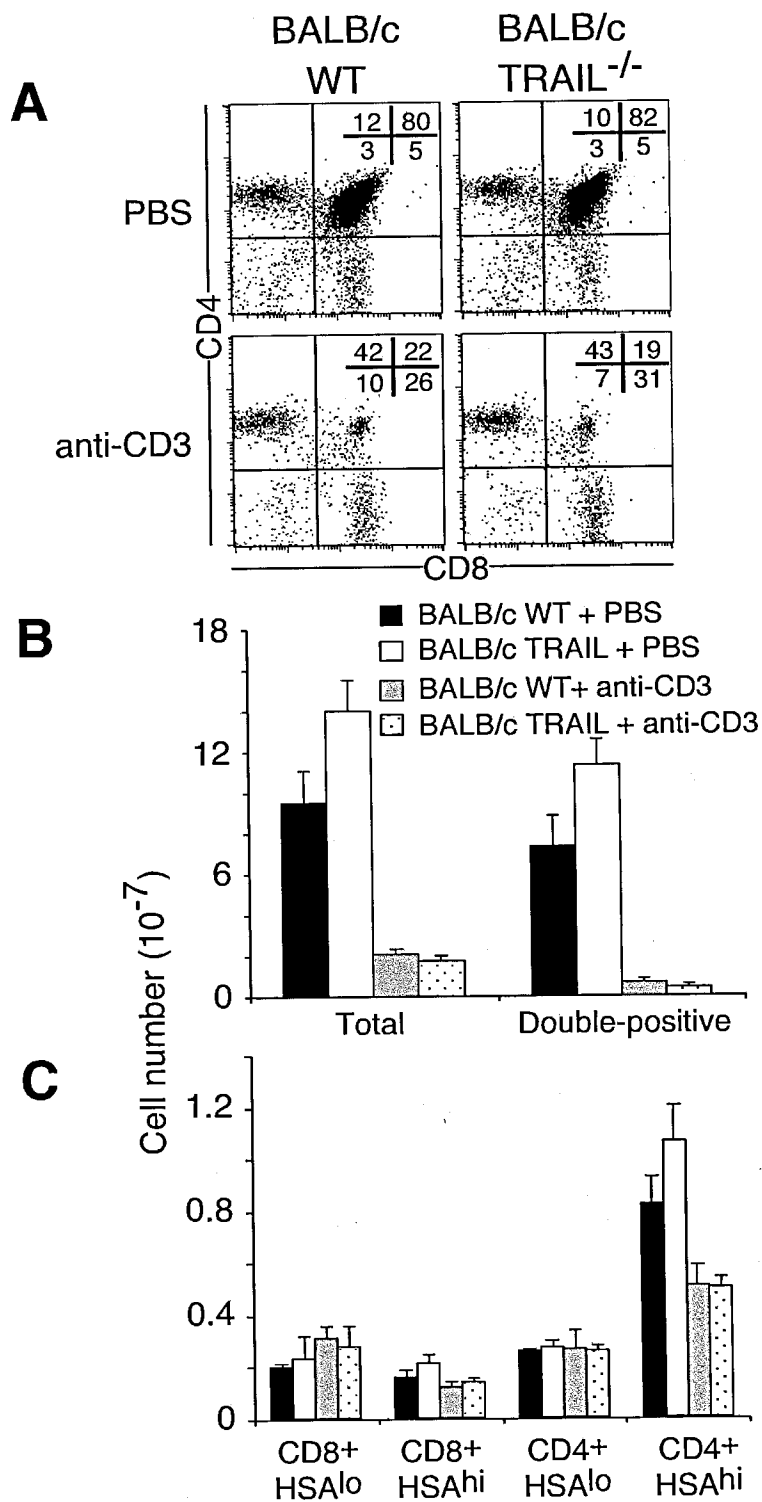


Figure 6.8. Anti-CD3 mAb mediated thymocyte apoptosis *in vivo*. BALB/c and BALB/c TRAIL^{-/-} mice were injected i.p. with either 20 μ g anti-CD3 mAb (145-2C11) or PBS vehicle control. Forty hours after injection, mice were sacrificed, their thymocytes and splenocytes isolated, counted and stained with antibodies to CD4, CD8, TCR $\alpha\beta$ and HSA (CD24). (A) Representative CD4 versus CD8 FACS profiles, (B) numbers of total and DP thymocytes and (C) total numbers of CD8⁺ and CD4⁺ HSA^{hi} and HSA^{lo} cells per thymus is shown. Mean \pm SEM were calculated from analysis of cells isolated from 3 BALB/c and 3 BALB/c TRAIL^{-/-} mice. Results are representative of three independent experiments.

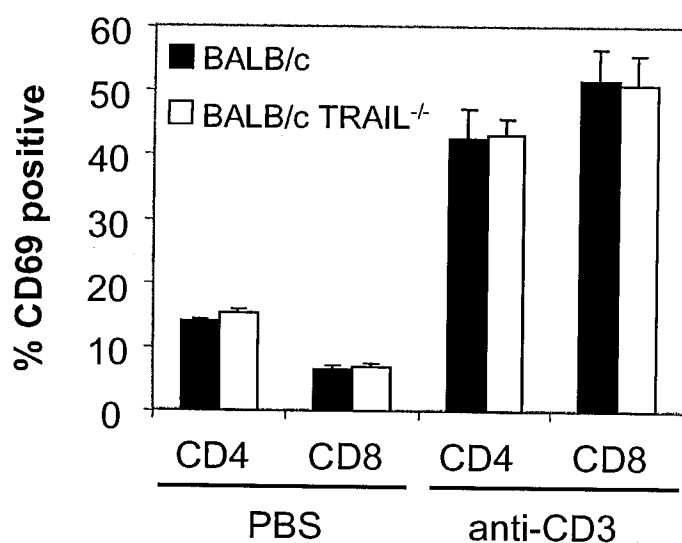


Figure 6.9. Equivalent peripheral activation in anti-CD3 treated BALB/c and BALB/c TRAIL-deficient mice. BALB/c and BALB/c TRAIL^{-/-} mice were injected i.p. with either 20 μ g anti-CD3 mAb (145-2C11) or PBS vehicle control. Forty hours after injection, mice were sacrificed, their splenocytes isolated and stained with antibodies to CD4, CD8 and CD69. CD69 staining on CD4⁺ and CD8⁺ splenocytes isolated from PBS or anti-CD3 mAb treated mice is shown. Mean \pm SEM are shown for results pooled from two experiments, each containing 3 or 4 BALB/c and 3 or 4 BALB/c TRAIL^{-/-} mice injected i.p. with PBS or anti-CD3 mAb. Percentage CD69⁺ cells was not significantly different for BALB/c and BALB/c TRAIL^{-/-} mice ($P > 0.05$).

critical for negative selection, TRAIL expression might be predicted on epithelial cells and/or DCs, and DR5 expression might be expected on target thymocyte populations. Thymic epithelial cells and DCs were isolated as described in Materials and Methods, Chapter 6, Section 6.3 and identified by flow cytometry as CD45⁺EpCAM⁺ and CD11c⁺MHC-II^{hi} respectively. TRAIL expression was not observed on thymic epithelial cells, DCs or thymocyte populations, although TRAIL was clearly expressed on liver NK cells as a positive control (data not shown) (Figure 6.10). Like peripheral DCs, thymic DCs expressed DR5 and some low level DR5 staining was observed on thymocytes, although the functional significance of this low level staining is yet to be determined (Figure 6.10). Levels of TRAIL and DR5 staining were unchanged in thymocytes activated with anti-CD3 and anti-CD28 mAb (Figure 6.11). Therefore, unless special *in vivo* conditions permit the expression of DR5 on thymocytes, and TRAIL on cells that can induce negative selection, it is unlikely that TRAIL can induce apoptosis within the thymus.

6.4.7 Aged C57BL/6 TRAIL-deficient mice do not develop spontaneous autoimmune disease

Defects in negative selection have been associated with the development of spontaneous autoimmune disease (Anderson et al., 2002; Bouillet et al., 1999; Bouillet et al., 2002; Liston et al., 2003). As negative selection is responsible for the removal of potentially autoreactive thymocytes, defects in negative selection might allow the release of self-reactive T cells into the peripheral pool, predisposing an individual to autoimmune disease.

As TRAIL-deficient mice were reported to exhibit defective negative selection (Lamhamedi-Cherradi et al., 2003), we aged C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice > 555 days to investigate whether TRAIL suppressed spontaneous autoimmune disease. Serum isolated from aged mice was analyzed for levels of total IgG (Figure 6.12A) and anti-dsDNA autoantibodies (Figure 6.12B.). Serum isolated from 28 week old C57BL/6 *gld* mice was used as a positive control, as these mice exhibit elevated levels of serum IgG and circulating autoantibodies (Korner et al., 2000). Neither total IgG nor anti-dsDNA autoantibodies

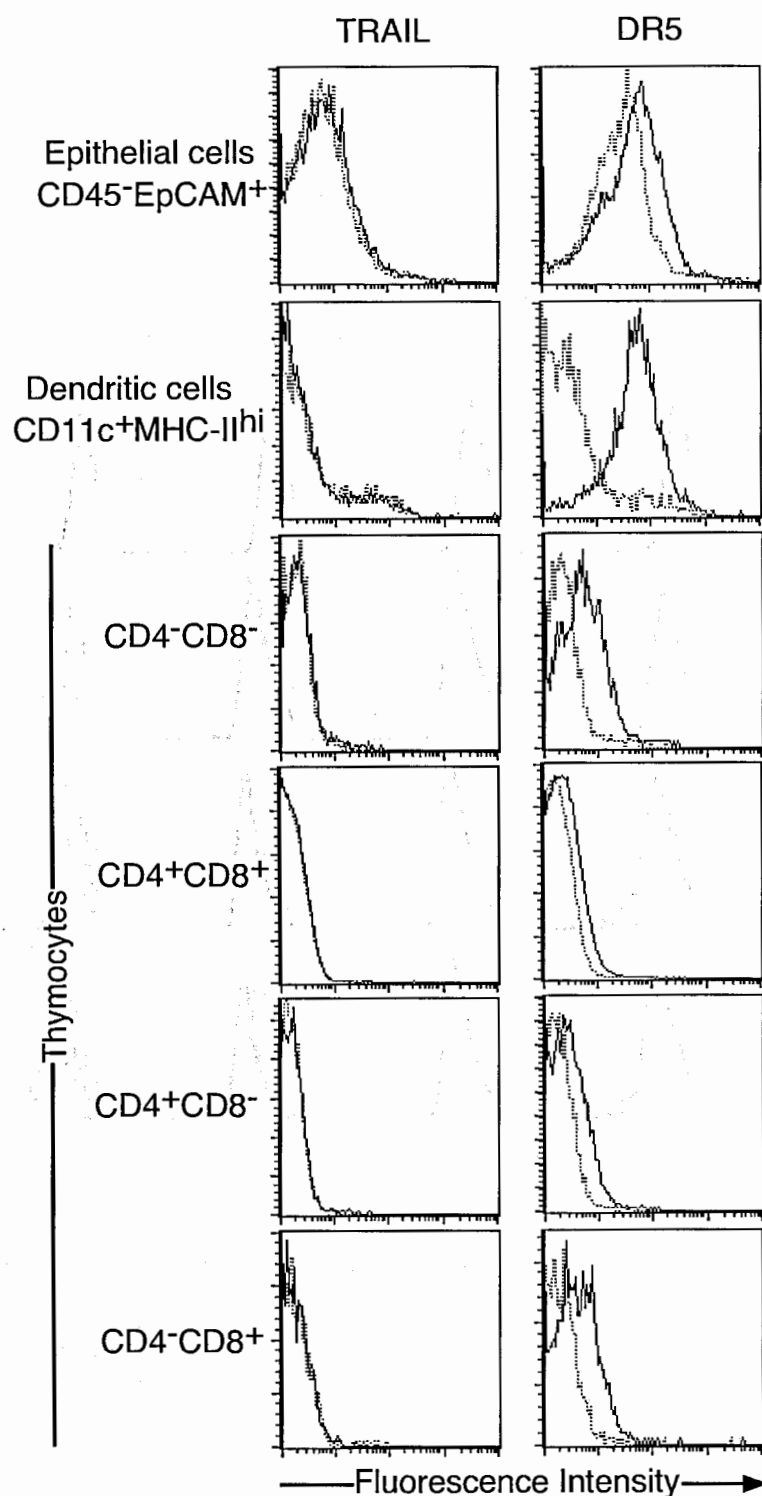


Figure 6.10. TRAIL and TRAIL receptor (DR5) expression within the thymus. Thymic epithelial cells, thymic DCs and thymocytes were isolated from BALB/c thymi (n=5) by enzymatic digestion with DNase and collagenase. Cells were analyzed by flow cytometry for TRAIL and TRAIL receptor (DR5) expression. Thymic epithelial cells were identified as CD45⁻EpCAM⁺, thymic DCs as CD11c⁺MHC-II^{hi} and thymocyte subsets were identified by staining with antibodies to CD4 and CD8. Dotted histograms represent isotype control staining and black histograms represent TRAIL or DR5 staining as indicated. Results are representative of three independent experiments (thymocytes) or one experiment (epithelial and DCs).

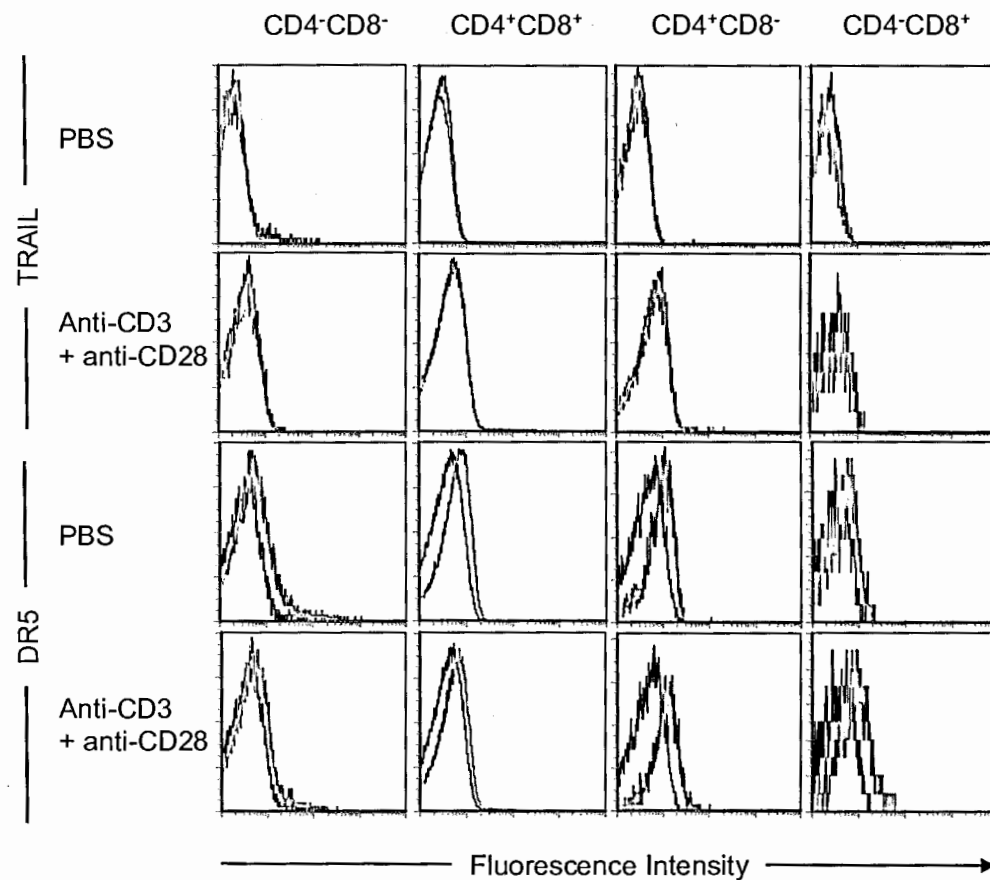
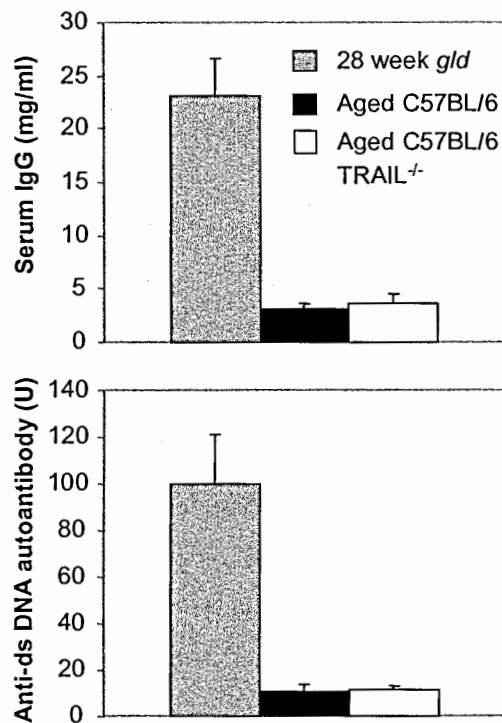


Figure 6.11. Expression of TRAIL and TRAIL receptor (DR5) by activated thymocytes. Thymocytes isolated from adult BALB/c and BALB/c TRAIL^{-/-} mice were cultured for 20 h in plates coated with 20 µg/ml anti-CD3ε (145-2C11) plus 10 µg/ml anti-CD28 (37.51) mAb or PBS. Activated thymocytes were then stained with antibodies to CD4, CD8, DR5 and mTRAIL. Dotted histograms represent isotype control staining and black histograms represent TRAIL or DR5 staining as indicated. Results are representative of three independent experiments, each containing 2-3 mice.



C

	Aged C57BL/6 (mean \pm SEM)	Aged C57BL/6 TRAIL ^{-/-} (mean \pm SEM)
aLN		
CD4 SP%	19.24 \pm 5.46	23.21 \pm 4.23
CD8 SP%	15.42 \pm 1.53	15.63 \pm 2.39
CD4 ⁺ CD8 ⁺ %	0.34 \pm 0.02	0.38 \pm 0.06
TCR- β SP%	52.37 \pm 6.98	44.37 \pm 4.73
B220 SP%	39.22 \pm 8.47	43.32 \pm 4.28
Spleen		
CD4 SP%	13.76 \pm 5.00	13.69 \pm 2.45
CD8 SP%	11.25 \pm 2.62	9.90 \pm 2.29
CD4 ⁺ CD8 ⁺ %	0.25 \pm 0.29	0.2 \pm 0.03
TCR- β SP%	37.28 \pm 11.7	41.33 \pm 8.63
B220 SP%	46.71 \pm 11.63	44.31 \pm 8.42
Thymus		
CD4 SP%	5.38 \pm 0.87	6.87 \pm 1.83
CD8 SP%	3.87 \pm 1.12	5.45 \pm 2.11
CD4 ⁺ CD8 ⁺ %	84.59 \pm 0.65	78.73 \pm 6.15
TCR- β SP%	8.39 \pm 2.76	9.88 \pm 3.08
B220 SP%	4.80 \pm 3.94	9.00 \pm 4.45

Figure 6.12. Serum antibody concentration and lymphoid organ composition in aged C57BL/6 and C57BL/6 TRAIL-deficient mice. Serum was isolated from aged (> 555 days) C57BL/6 and C57BL/6 TRAIL^{-/-} or 28 week old C57BL/6 *gld* mice (as a positive control) and tested by ELISA for (A) total concentration of IgG and (B) anti-dsDNA autoantibodies. The concentration of anti-dsDNA autoantibodies is shown relative to that observed for 28 week old C57BL/6 *gld* mice (100 Units was defined as the mean \pm SEM of absorbance at 405 nm for 28 week C57BL/6 *gld* mice). Mean \pm SEM were calculated from the sera of 3 C57BL/6, 3 C57BL/6 TRAIL^{-/-} and 4 C57BL/6 *gld* mice. (C) The lymphocyte composition observed in the aLN, spleen and thymus of aged C57BL/6 and C57BL/6 TRAIL^{-/-} mice is also shown. SP, single positive. Mean \pm SEM were calculated from analysis of cells isolated from 4 C57BL/6 and 6 C57BL/6 TRAIL^{-/-} mice. Results are pooled from two independent experiments. Serum IgG, anti-dsDNA and lymphocyte composition are not significantly different for aged C57BL/6 and C57BL/6 TRAIL^{-/-} mice ($P > 0.05$).

were elevated in aged C57BL/6 WT or C57BL/6 TRAIL^{-/-} mice (Figure 6.12 A and B). Moreover, the overall lymphocyte composition was comparable in LN, spleen and thymus of aged C57BL/6 WT and C57BL/6 TRAIL^{-/-} mice (Figure 6.12C) and histological analysis of a number of organs failed to reveal any obvious signs (such as lymphocytic infiltration or tissue destruction) of spontaneous autoimmune disease in these mice (Figure 6.13). Collectively these findings suggest that TRAIL does not suppress spontaneous autoimmune disease.

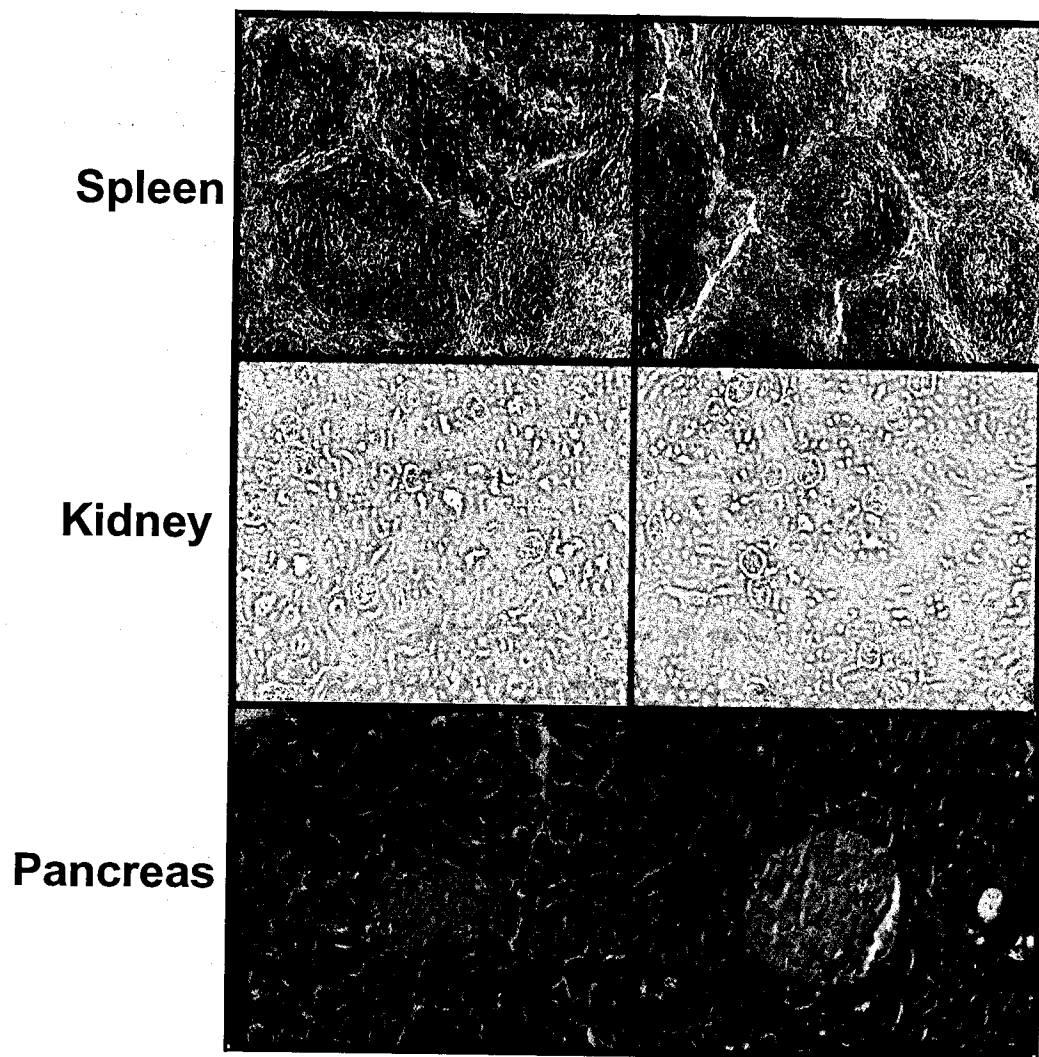


Figure 6.13. Aged C57BL/6 TRAIL-deficient mice do not develop spontaneous autoimmune disease. Representative H&E staining on spleen, kidney and pancreas sections isolated from C57BL/6 (A, C, E) and C57BL/6 TRAIL^{-/-} (B, D, F) mice aged 555-700 days. Normal spleen, kidney and pancreatic architecture with a lack of obvious signs of spontaneous autoimmune disease are observed in aged C57BL/6 and C57BL/6 TRAIL^{-/-} mice. Original magnification x10 for spleen and kidney and x 20 for pancreas.

6.5 Discussion

The role of TRAIL signaling in intrathymic negative selection is controversial. A recent report by Lamhamedi-Cherradi and colleagues (Lamhamedi-Cherradi et al., 2003) suggested that TRAIL was critical for negative selection of autoreactive thymocytes. Our data, using five separate models of thymocyte negative selection, suggested that TRAIL was not required for this process. Thus, our data concurs with an earlier study using soluble blocking TRAIL-R2-Fc to demonstrate that negative selection of mouse and human thymocytes was TRAIL-independent (Simon et al., 2001). Moreover, our data is in agreement with results from Smith et al. (Smith et al., 1996) and Newton et al. (Newton et al., 1998) that clearly exclude a role for the FADD-caspase 8 pathway (which is essential for TRAIL-R and DR-induced apoptosis in general) in negative selection of T cells.

It is difficult to explain the discrepancies between our results and those recently published (Lamhamedi-Cherradi et al., 2003), which use TRAIL^{-/-} mice from the same source and similar models to investigate negative selection. However, careful analysis of at least some of the data in the Lamhamedi-Cherradi et al. study reveals some uncertainties. Proportions of TCRV β 5⁺ and TCRV β 11⁺ T cells in peripheral lymphoid organs in TRAIL^{-/-} mice are only slightly (albeit significantly) increased (Lamhamedi-Cherradi et al., 2003). Cells with these specificities are deleted by the endogenous superantigen MMTV9 presented by I-E⁺ class II MHC as occurs in BALB/c mice. However, proportions of these cells in I-E⁻ mice such as C57BL/6 WT, are at least 10 times that detected in BALB/c WT mice. Given that the percentage of TCRV β 5⁺ and TCRV β 11⁺ T cells in BALB/c TRAIL^{-/-} was only slightly higher than that observed in control BALB/c WT mice, the impact of TRAIL in this model of negative selection appears to be minimal at best. Moreover, DP rather than SP thymocytes were analyzed for MMTV-mediated negative selection (Lamhamedi-Cherradi et al., 2003). Analysis of DP thymocytes is complicated, as most of these cells express low levels of TCR $\alpha\beta$ and only the very latest stages of DP thymocyte development are susceptible to MMTV-mediated negative selection (Guidos et al., 1990; Hugo et

al., 1991). Thus, changes in TCRV β representation are far easier and more reliable to detect in SP thymocytes. In fact, our own data and that of others (Hugo et al., 1991), suggests that whilst TCRV β ⁵⁺ T cells are clearly deficient at the SP stage, these cells are not dramatically under-represented in the DP thymocyte population. Further uncertainties were revealed in the analysis of C57BL/6 TRAIL^{-/-} mice. MMTV9 can mediate significant deletion of TCRV β ⁵⁺ cells in C57BL/6 WT mice (I-A⁺, I-E⁻) and the proportion of these cells is increased in Bim-deficient mice that are known to have defective negative selection. However, no difference in the proportions of these cells were reported in C57BL/6 TRAIL^{-/-} mice, again raising some concern over the extent to which TRAIL regulates negative selection (Lamhamedi-Cherradi et al., 2003).

An important finding in this study was that thymic DCs and thymic epithelial cells which are the two most important cellular mediators of negative selection in the thymus (Hoffmann et al., 1995; Sprent and Webb, 1995; Tanaka et al., 1993), do not constitutively express TRAIL. Thymocyte populations also lacked TRAIL expression. If TRAIL was critical for negative selection, TRAIL expression might be predicted on these cells. Although TRAIL RNA has previously been detected in whole thymus (Wiley et al., 1995), this would likely reflect the presence of thymocyte rather than stromal cell TRAIL RNA given that thymocytes comprise 99% of thymus cellularity. Nonetheless, if TRAIL expressing cells capable of mediating negative selection are present in the thymus, TRAIL receptor (DR5) expression would be required for induction of an apoptotic signal in thymocytes. Our extensive flow cytometric analysis revealed extremely low levels of DR5 expression on thymocytes. DR5 expression was unchanged when thymocytes were cultured *in vitro* with anti-CD3 and anti-CD28 mAb. The level of DR5 expression detected on thymocytes was approximately 10 to 100-fold lower than typical DR5 expression detected on TRAIL-sensitive tumor cell lines or on primary cells such as DCs. To clarify whether the low level DR5 expression on thymocytes is physiologically relevant, functional assays are required to determine the sensitivity of mouse thymocytes to TRAIL killing. Human thymocytes have previously been shown to express low levels of TRAIL-R1 and R2 and to become sensitized to TRAIL-induced apoptosis upon

stimulation with anti-CD3 mAb (Simon et al., 2001), although this is yet to be examined for mouse thymocytes. Thus, the physiological relevance of DR5 expression in the thymus in the absence of TRAIL expressing effector cells is questionable. It will be interesting to further assess negative selection in mice lacking DR5.

As negative selection is responsible for the removal of potentially autoreactive thymocytes, defects in negative selection might allow the release of self-reactive T cells into the peripheral pool, predisposing an individual to autoimmune disease. Deficiency of the pro-apoptotic Bcl-2 family member Bim, that is critical for negative selection, leads to spontaneous development of autoimmune glomerulonephritis (Bouillet et al., 1999). Mutation of AIRE inhibits the expression of peripheral antigens in the thymus, preventing the deletion of self-reactive T cells, and predisposes mice (Anderson et al., 2002; Liston et al., 2003) and humans (Nagamine et al., 1997) to a multitude of organ-specific autoimmune diseases. We failed to reveal any signs of spontaneous autoimmunity in aged C57BL/6 TRAIL^{-/-} mice (> 555 days), compared with aged syngeneic C57BL/6 WT mice. Perhaps this is the strongest argument that TRAIL is not critical for maintaining self-tolerance, and is dispensable for intrathymic negative selection. In Chapter 5, TRAIL was clearly demonstrated to suppress autoimmune damage in three different models of EAE. Others studies have also demonstrated that TRAIL-deficient mice are more susceptible to the induction of autoimmunity in certain experimental models (Hilliard et al., 2001; Lamhamedi-Cherradi et al., 2003; Song et al., 2000), however, it remains unclear by what mechanism TRAIL suppresses the induction of autoimmunity. Clearly, disease induction in these experimental models is complex and often involves adjuvants that stimulate DCs, innate immune cell networks that may involve TRAIL function, or AICD in the periphery. Indeed, several studies have implicated TRAIL in AICD (Martinez-Lorenzo et al., 1998; Zhang et al., 2003), although it remains to be determined whether TRAIL contributes to the maintenance of self-tolerance in this manner.

Whilst TRAIL may be functional on activated mature T cells and NK cells and may be capable of suppressing induction of experimental autoimmune disease (as

illustrated in Chapter 5), we have shown in five different models that TRAIL is not critical for intrathymic negative selection. We are unable to easily explain the basis of the discrepancies between our results and those recently published (Lamhamedi-Cherradi et al., 2003), but in light of our data and other supportive studies (Simon et al., 2001) we suggest that the importance of TRAIL in intrathymic negative selection is, at best, unclear and requires further investigation. Rigorous testing is now required to determine whether TRAIL can induce apoptosis in a FADD-caspase 8-independent, but Bim-dependent manner.

CHAPTER SEVEN

GENERAL DISCUSSION

Important research completed prior to this thesis had focused on investigating the potential of rTRAIL as a cancer therapeutic since it demonstrated a unique ability to induce apoptosis in transformed cells, but not in most normal cells (Ashkenazi, 2002; Ashkenazi et al., 1999; Walczak et al., 1999). Despite great interest in TRAIL as a single agent or in combination with chemotherapeutic drugs (Ashkenazi et al., 1999; Nagane et al., 2000) or ionizing radiation (Chinnaiyan et al., 2000) for cancer therapy, little was known about TRAIL protein expression, or the function of TRAIL in immune responses and tumor immunosurveillance. This thesis provides the first detailed evidence of the leukocyte expression and function of mouse TRAIL. Importantly, we have demonstrated that TRAIL suppresses the initiation, growth and metastasis of tumors in mice, and that TRAIL inhibits EAE induction, but does not play a role in thymic negative selection of T cells or regulation of lymphoproliferation in Fas ligand mutant mice. Collectively, this data suggested that appropriate targeted manipulation of the TRAIL pathway might prove to be a useful strategy to suppress tumor growth and T cell-mediated autoimmune disease.

7.1 Constitutive TRAIL expression in the mouse

Previously, our laboratory had shown that TRAIL was constitutively expressed on a small subset of liver NK cells in adult mice (Takeda et al., 2001). Herein, we have extended the earlier study to demonstrate that TRAIL is expressed on a unique population of phenotypically immature NK cells in the adult liver. Moreover, phenotypically similar TRAIL-expressing NK cells predominate in the liver and spleen of fetal and neonatal mice, but decrease in proportion with age and are uniquely retained in decreased numbers in the adult liver. Several possibilities exist as to why TRAIL⁺ NK cells are retained in the adult liver and not in other lymphoid organs. TRAIL might play a role in lymphocyte cell death that is reported to occur in the liver (Eberl and MacDonald, 1998; Mehal et al., 1999), or the surveillance of stressed cells in this site (Rabinovich et al., 2000), however there was no abnormal accumulation of lymphocytes observed in TRAIL-deficient mice. TRAIL might also play a role in tolerance responses to antigens derived from food or endogenous commensural pathogens that may first

traffic from the gut via the portal vein. Interestingly, Takeda and colleagues have recently shown that germ-free mice also contain the same TRAIL⁺ population of adult liver NK cells (personal communication, Dr. Kazuyoshi Takeda). Alternatively, TRAIL might play a role in NK cell-mediated clearance of pathogens within the liver. IFN- γ produced by liver NK cells plays an important role in the regulation of MCMV infection (Tay and Welsh, 1997), and human foreskin fibroblasts infected with HCMV upregulate expression of DD-containing TRAIL receptors and become sensitive to TRAIL-mediated apoptosis (Sedger et al., 1999). Therefore, it might now be interesting to examine whether TRAIL expressed on liver NK cells can regulate CMV infection in the liver. Future studies to investigate the role of TRAIL in the regulation of malaria infection might also be warranted, given that IFN- γ production by NK cells is important in the regulation of the sporozoite stage of malaria infection in the liver (Mohan et al., 1997; Ojo-Amaize et al., 1984).

Importantly, we have shown that TRAIL is the first effector molecule expressed on fetal NK cells. More recently, our collaborators have shown that adoptive transfer of immature TRAIL⁺ NK cells isolated from neonatal mice or adult liver resulted in the appearance of TRAIL⁻ NK cells with a mature phenotype (personal communication, Dr. Kazuyoshi Takeda). This suggested that TRAIL⁺ NK cells are the precursors of TRAIL⁻ NK cells. Our data suggests that TRAIL is a distinctive functional marker to define a unique stage of NK cell differentiation. Expression of high levels of CD94 by fetal NK cells has been suggested to play an important role in the maintenance of self-tolerance during fetal life (Salcedo et al., 2000; Sivakumar et al., 1999; Van Beneden et al., 2001). Thus, the high level of CD94 expression observed on TRAIL⁺ liver NK cells might act to negatively regulate these cells, preventing cytotoxicity against self-tissue *in vivo*. Our study suggests that the limited availability of NK cell effector pathways early in life might comprise another level of regulation. TRAIL might be the preferred effector molecule pathway for potentially self-reactive NK cells when they lack the majority of MHC class I binding inhibitory receptors (eg. Ly49s).

Understanding the specific cytokines that regulate constitutive TRAIL expression *in vivo* might ultimately enable therapeutic manipulation of TRAIL function *in vivo*. Indeed, our finding that IFN- γ regulated constitutive TRAIL expression *in vivo* prompted further studies in our laboratory which revealed that TRAIL induction on NK cells played a critical role in the IFN- γ -mediated anti-metastatic effects of α -GalCer and IL-12 (Smyth et al., 2001). Our laboratory is now investigating the effects of other biological response modifiers such as IL-18 and IL-21, that induce NK cell IFN- γ production, on TRAIL function.

7.2 TRAIL and T cell homeostasis

Several years ago, our laboratory reported a critical role for TNF in the lymphoaccumulation and premature death associated with the *gld* phenotype (Korner et al., 2000). Given that TRAIL can induce apoptosis in activated T and B cells (Marsters et al., 1996; Ursini-Siegel et al., 2002; Wang et al., 2000), we hypothesized that TRAIL, like TNF, might regulate lymphoproliferation in *gld* mice. Surprisingly, TRAIL had no effect on the *gld* phenotype. The role of TRAIL in T cell homeostasis was additionally investigated using several models of thymocyte negative selection. Although TRAIL had previously been reported to be critical for intrathymic negative selection (Lamhamedi-Cherradi et al., 2003b), we clearly showed that negative selection is normal in TRAIL-deficient mice. We additionally showed that negative selection is normal in mice deficient in both TRAIL and FasL, suggesting that TRAIL and FasL do not function cooperatively to induce negative selection. It might now be of interest to delete multiple TNF-superfamily members, to determine whether negative selection is completely independent of DRs. Although we can not rule out a specific role for TRAIL in T cell regulation, together our data suggested that TRAIL does not globally affect T cell homeostasis.

7.3 TRAIL and autoimmune disease

TRAIL has been shown to inhibit autoimmune disease in a variety of mouse models including autoimmune arthritis (Lamhamedi-Cherradi et al., 2003b; Song

et al., 2000) and autoimmune diabetes (Lamhamedi-Cherradi et al., 2003b) (Lamhamedi-Cherradi et al., 2003a). In these studies, TRAIL was suggested to play a variety of roles ranging from inhibiting cytokine and antibody production to inhibiting inflammation and cell cycle progression. The data presented in this thesis have clearly demonstrated that TRAIL suppresses autoimmune damage in relapsing-remitting and chronic non-remitting models of EAE. Our data suggested that TRAIL suppressed EAE by inhibiting T cell proliferation and infiltration of leukocytes into the CNS. These findings substantiated an important and general role for TRAIL in the inhibition of the induction of T cell-mediated autoimmune disease.

Disease induction in experimental models of autoimmune disease is extremely complex and often involves adjuvants that stimulate DCs and other innate immune cells and activation-induced T cell death in the periphery. Given that TRAIL expression can be induced on activated NK cells (Kayagaki et al., 1999) and DCs (Fanger et al., 1999), the involvement of TRAIL in EAE might be attributed to the effects of adjuvant administered to induce disease. Indeed, CFA administration is known to stimulate NK cell IFN- γ production (Lee et al., 2004; Shi et al., 2000), and to suppress autoimmune diabetes (Lee et al., 2004) and pristane-induced arthritis in mice (Zheng et al., 2002; Zheng et al., 2003). Moreover, studies in our laboratory have implicated autocrine IFN- γ produced by NK cells in the regulation of TRAIL expression (Takeda et al., 2001). Collectively, these findings entertain the possibility that TRAIL-mediated suppression of EAE might be adjuvant-dependent.

The precise mechanism(s) whereby TRAIL suppresses EAE, and the key TRAIL-expressing effector cells involved are unknown. Although TRAIL can suppress EAE, our study revealed that aged C57BL/6 TRAIL^{-/-} mice do not develop spontaneous autoimmune disease, suggesting a minor role at best for TRAIL in suppression of autoimmune disease. Importantly, we disproved the postulate that TRAIL plays a key role in negative selection of thymocytes, suggesting that TRAIL inhibits EAE induction by a different mechanism. Immunofluorescence studies using the anti-mTRAIL mAb might help elucidate the key TRAIL-

expressing effector cells that infiltrate the CNS in EAE. NK cells are a good effector cell candidate to express TRAIL, and have been established to suppress EAE in mice (Zhang et al., 1997). Future NK cell depletion studies in TRAIL-deficient mice are required to determine whether TRAIL-mediated suppression of EAE is NK cell-dependent. Our laboratory have recently shown that TRAIL-expressing NK cells can eliminate DCs *in vivo* (Hayakawa et al., 2004), leading us to speculate that TRAIL-expressing NK cells might suppress EAE by inducing apoptosis in DCs that prime autoreactive T cells. Alternatively, TRAIL-expressing NK cells might induce apoptosis in autoreactive T cells to suppress disease.

DCs or autoreactive T cells might also potentially express TRAIL and suppress EAE. Indeed, recent studies have suggested that TRAIL-expressing DCs can inhibit proliferation of autoreactive T cells in EAE to suppress disease (Suter et al., 2003). Alternatively, TRAIL-expressing DCs might be hypothesized to directly induce apoptosis in autoreactive T cells. EAE can be induced in naïve mice by transfer of BM-derived DCs pulsed with MOG peptide *in vitro* (Weir et al., 2002). If DCs suppress EAE in a TRAIL-dependent manner, transfer of DCs isolated from TRAIL^{-/-} mice might be expected to induce a more severe form of EAE. If autoreactive T cells can suppress EAE in a TRAIL-dependent manner, adoptive transfer of autoreactive T cells isolated from TRAIL-deficient TCR transgenic mice (specific for MBP, PLP or MOG) may induce a more severe form of EAE compared to transfer of TRAIL-sufficient autoreactive T cells. Clearly, understanding how TRAIL suppresses EAE will be of great importance before any future planning of clinical trials in MS patients using rTRAIL/Apo2L.

In this thesis, we have illustrated the therapeutic value of rTRAIL and anti-DR5 antibody in suppressing the animal model EAE. Recent studies in our laboratory have shown that although rTRAIL or anti-DR5 antibody administration from the time of disease induction could suppress MOG-induced EAE in NOD/Lt mice, treatment during the disease course had no effect on the severity of EAE. Importantly, these data suggested that early treatment with rTRAIL or anti-DR5 antibody is required to maximally exploit the TRAIL-TRAIL-R pathway to

therapeutic advantage. This highlights the importance of early detection of disease if rTRAIL or humanized anti-DR5 antibody is to be used effectively to treat MS patients. In a recent study, TRAIL was identified as a response marker for IFN- β therapy in MS patients (Wandinger et al., 2003). Patients with raised levels of TRAIL in their sera prior to IFN- β therapy and those showing sustained TRAIL induction post-therapy responded best to treatment (Wandinger et al., 2003). Although this report suggested that TRAIL might be effective in MS patients, a number of therapies that are effective in suppression of EAE are ineffective in MS patients (Pender and Wolfe, 2002), and the TRAIL receptor system in humans differs from mice. Once TRAIL is shown to be safe in Phase I trials in cancer patients, clinical trials may be considered to determine the therapeutic efficacy of targeting the TRAIL pathway in MS patients.

7.4 TRAIL and tumor immune surveillance

Previously, a number of studies had demonstrated the ability of rTRAIL to induce regression of tumor xenografts in mice without systemic toxicity (Ashkenazi et al., 1999; Walczak et al., 1999), although less was known about the role TRAIL played in natural tumor immunity. The studies in this thesis have supported a direct role for TRAIL in suppression of tumor initiation and metastasis. Importantly, we have reported the initial characterization of TRAIL gene-targeted mice, and identified a number of TRAIL-sensitive mouse tumor models that will facilitate in depth studies investigating the role of TRAIL in tumor immunity.

The mammary gland is a common site of human neoplasia. In this study we uniquely demonstrated that TRAIL suppressed tumor growth in the mammary gland and metastases from this site. This finding suggested that TRAIL might be constitutively expressed on NK cells within the mammary gland, or that a presence of transformed cells in this site might stimulate an infiltration of TRAIL-expressing NK cells. We are now establishing TRAIL-deficient Her2/neu transgenic mice to investigate whether TRAIL plays a general role in suppression of tumor growth in the mammary gland. Given that IFN- γ might play a role in control of tumor development in Her2/neu transgenic mice (Boggio et al., 1998),

and IFN- γ is important in the regulation of TRAIL expression *in vivo*, these studies are of particular interest.

In addition to the studies described in this thesis, a number of recent studies in our laboratory and others have greatly contributed to our knowledge of the role TRAIL plays in tumor surveillance. TRAIL was shown to play a role in natural protection from spontaneous tumor development in C57BL/6 p53^{+/-} mice (Takeda et al., 2002). Importantly, TRAIL was also demonstrated to suppress the initiation of MCA-induced fibrosarcomas (Cretney et al., 2002). The majority of fibrosarcoma cell lines established from MCA-inoculated TRAIL^{-/-} or anti-mTRAIL mAb-treated mice were susceptible to TRAIL-mediated cytotoxicity, whereas the majority of fibrosarcomas isolated from control-Ig-treated mice were TRAIL-resistant (Takeda et al., 2002). This suggested that TRAIL might naturally select TRAIL-resistant tumor variants. Future studies in our laboratory will investigate the mechanisms by which tumors escape TRAIL-mediated tumor surveillance. TRAIL-resistant MCA-induced fibrosarcoma cell lines will be assessed for DR5 and TRAIL decoy receptor expression, and key points in the cell death cascade normally triggered by TRAIL will be analyzed. By understanding the mechanism tumors use to escape TRAIL-mediated surveillance, we hope to design more effective therapies to suppress tumor initiation and metastasis *in vivo*.

A number of recent studies have helped elucidate the mechanisms whereby tumor cells become resistant to TRAIL-mediated apoptosis. TRAIL-sensitive tumor cells transfected with cFLIP were recently demonstrated to escape TRAIL-mediated surveillance *in vivo* (Seki et al., 2003), and inhibition of cFLIP with small interfering RNAs sensitized tumor cells to TRAIL-mediated apoptosis *in vitro* (Siegmond et al., 2002). Proteasome inhibitors such as PS-341 and N-acetyl-L-leuciny-L-leuciny-L-norleucinal (LLnL) have been reported to sensitize tumor cells to TRAIL-mediated apoptosis by reducing the levels of cFLIP (Sayers et al., 2003), blocking NF κ B activation (Franco et al., 2001; Jeremias et al., 1998) and elevating the levels of TRAIL receptors DR4 and DR5 (Johnson et al., 2003). Recently, a small molecule Smac mimic has been

developed that functions by relieving IAP-mediated suppression of caspase activity, and acts synergistically with TRAIL to induce tumor cell apoptosis (Li et al., 2004). In another report, overexpression of the *MYC* oncogene was reported to sensitize cells to TRAIL- or DR5 agonist-induced apoptosis, by upregulating cell surface expression of DR5 (Wang et al., 2004). These studies illustrate that sensitizing tumor cells to TRAIL-mediated apoptosis via modulation of various molecular targets, and specifically stimulating apoptosis using rTRAIL, may be an effective therapeutic approach.

The majority of the models used to investigate the role of TRAIL in tumor surveillance have been models of NK cell suppression. Recently, a significant role for TRAIL in T cell-mediated tumor immunity was reported (Schmaltz et al., 2002). TRAIL was required for optimal graft-versus-tumor activity by donor T cells in allogeneic hematopoietic-cell transplantation studies in mice (Schmaltz et al., 2002). Importantly, this study suggested that strategies to stimulate TRAIL expression and function on donor T cells might decrease relapse in leukemia patients. Recently, TRAIL expression and anti-tumor activity were reported to be induced in human B cells and macrophages by specific CpG-containing oligodeoxynucleotides (Kemp et al., 2003; Kemp et al., 2004). Given that *Mycobacterial* DNA contains high levels of CpG motifs, these studies prompted the investigation of the anti-tumor properties of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). BCG is a potent immunostimulant that induces a Th1 cytokine response and is an often prescribed treatment for patients with bladder cancer. Patients responding to BCG therapy exhibited higher levels of TRAIL in their urine compared to non-responders (Ludwig et al., 2004). Moreover, soluble urinary TRAIL could induce apoptosis in bladder carcinoma cells *in vitro* (Ludwig et al., 2004). Interestingly, while the role of NK cells was not specifically examined, TRAIL was shown to be expressed on voided neutrophils, suggesting an anti-tumor role for neutrophils *in vivo* (Ludwig et al., 2004). Together these studies indicated that a wide variety of effector cells might express TRAIL and mediate TRAIL-dependent anti-tumor activity *in vivo*.

TRAIL is primarily expressed as a type II transmembrane protein, but also exists as a soluble form that is enzymatically cleaved from the cell surface (Mariani and Krammer, 1998), or secreted in microvesicles (Monleon et al., 2001). The therapeutic efficacy of BCG correlates with an increased production of soluble TRAIL in patients with bladder cancer (Ludwig et al., 2004). Moreover, neutrophils and PBMCs isolated from CML patients and stimulated with IFN- α *in vitro* secrete high levels of soluble TRAIL, that induces apoptosis in TRAIL-sensitive leukemia cells *in vitro* (Tecchio et al., 2004). Collectively, these studies suggest that both soluble and membrane-bound TRAIL might be capable of exerting tumoricidal activity. It might now be interesting to produce mice deficient in membrane-bound, or soluble TRAIL, to help elucidate the importance of these forms of TRAIL in immunosurveillance. Indeed, mice deficient in soluble TNF have been used to differentiate the specific roles of membrane versus soluble TNF in mice (Ruuls et al., 2001).

7.5 Cancer therapeutic potential of TRAIL

Administration of rTRAIL in mice and primates induced tumor regression without systemic toxicity (Ashkenazi et al., 1999; Walczak et al., 1999) and TRAIL has been shown to act synergistically with ionizing radiation (Chinnaiyan et al., 2000) and chemotherapeutic drugs (Ashkenazi et al., 1999; Nagane et al., 2000). Radiation and most DNA-damaging chemotherapeutic drugs induce tumor cell apoptosis via p53-mediated activation of the intrinsic mitochondrial death pathway (Levine, 1997). In contrast, TRAIL induces apoptosis in a p53-independent manner via 'cell-extrinsic' or 'cell-intrinsic' death pathways (Ashkenazi et al., 1999). Treatment with rTRAIL might therefore be expected to circumvent resistance to conventional chemotherapy and radiotherapy in cancer patients. Genentech Incorporated is currently planning a Phase I clinical trial (PRO1762) of recombinant soluble TRAIL/Apo2L in cancer patients. The data in this thesis have confirmed that natural TRAIL plays an important role in tumor surveillance, providing hope that synthetic TRAIL will prove effective in the eradication of tumor in cancer patients.

A number of tumor xenograft studies have demonstrated the capability of agonistic mAbs that engage human DR4 or DR5 to exert anti-tumor activity *in vivo* (Chuntharapai et al., 2001; Ichikawa et al., 2001; Wang et al., 2004). Human Genome Sciences and Cambridge Antibody Technology are currently planning a Phase I clinical trial of an agonistic humanized anti-DR5 mAb in cancer patients. Specific targeting of death-inducing DR5 or DR4 receptors by agonistic antibodies might be advantageous over rTRAIL administration if tumor cells are protected from rTRAIL-induced apoptosis by the expression of cell surface decoy receptors. However, if normal cells over express decoy receptors as a protective mechanism against TRAIL-mediated killing, administration of agonistic antibodies to DR4 or DR5 might potentially prove more toxic than administration of rTRAIL.

The findings in this thesis have established an important role for TRAIL in tumor surveillance *in vivo* and have provided supporting rationale for the testing of soluble rTRAIL as a cancer therapeutic. Immunotherapies that mobilize TRAIL⁺ effector cells (including NK, T, myeloid cells and neutrophils) might prove effective in cancer patients. Given that rTRAIL acts synergistically with chemotherapy and radiotherapy, combinations of these therapies with TRAIL-inducing agents such as α -GalCer, IL-12 and IFNs might also prove effective.

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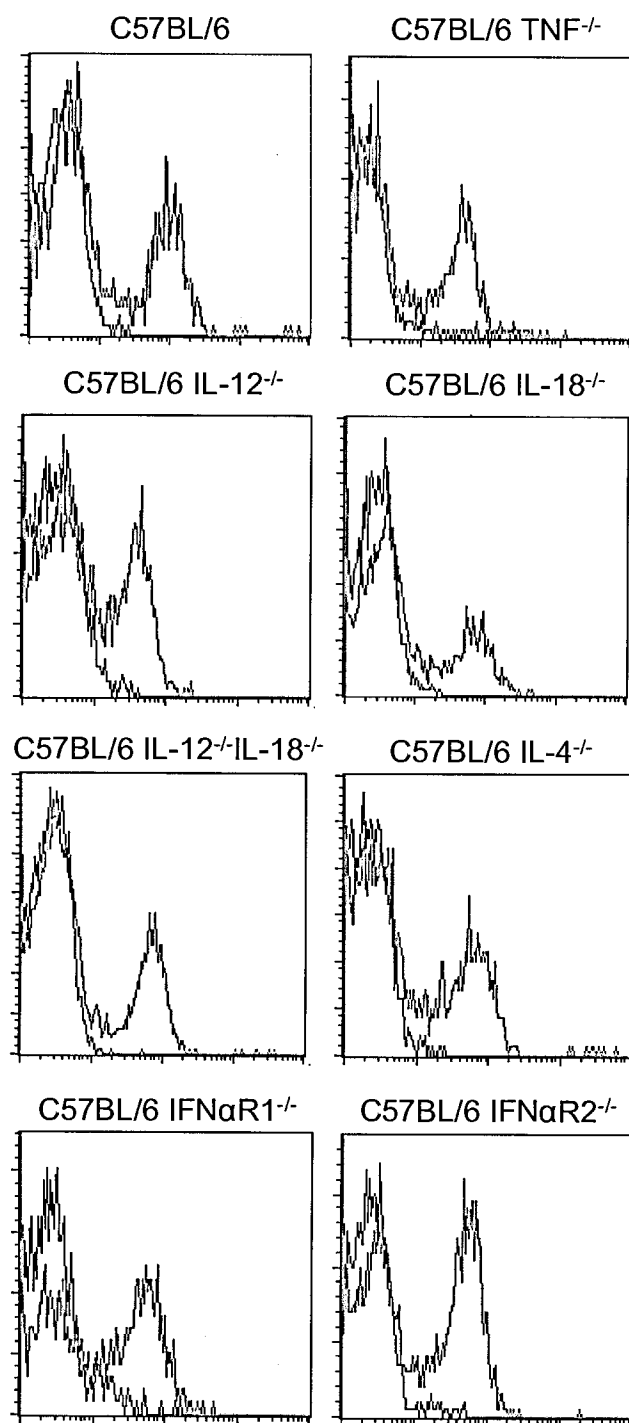
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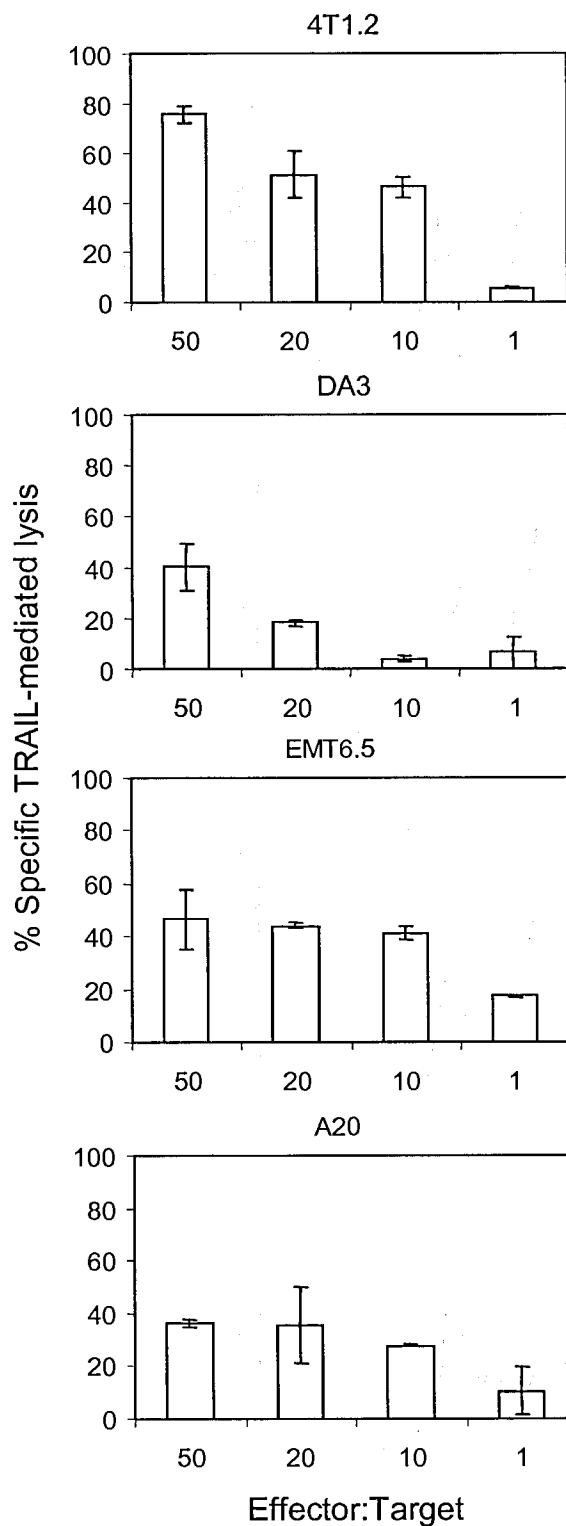
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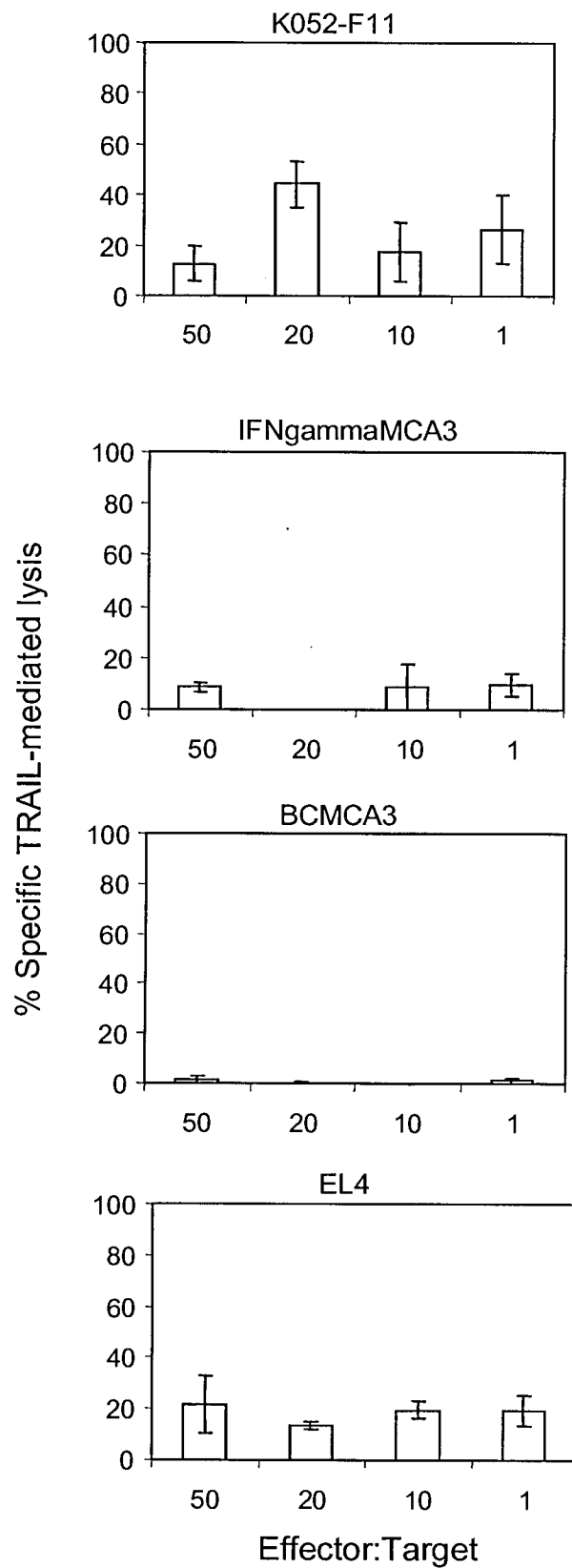
APPENDICES

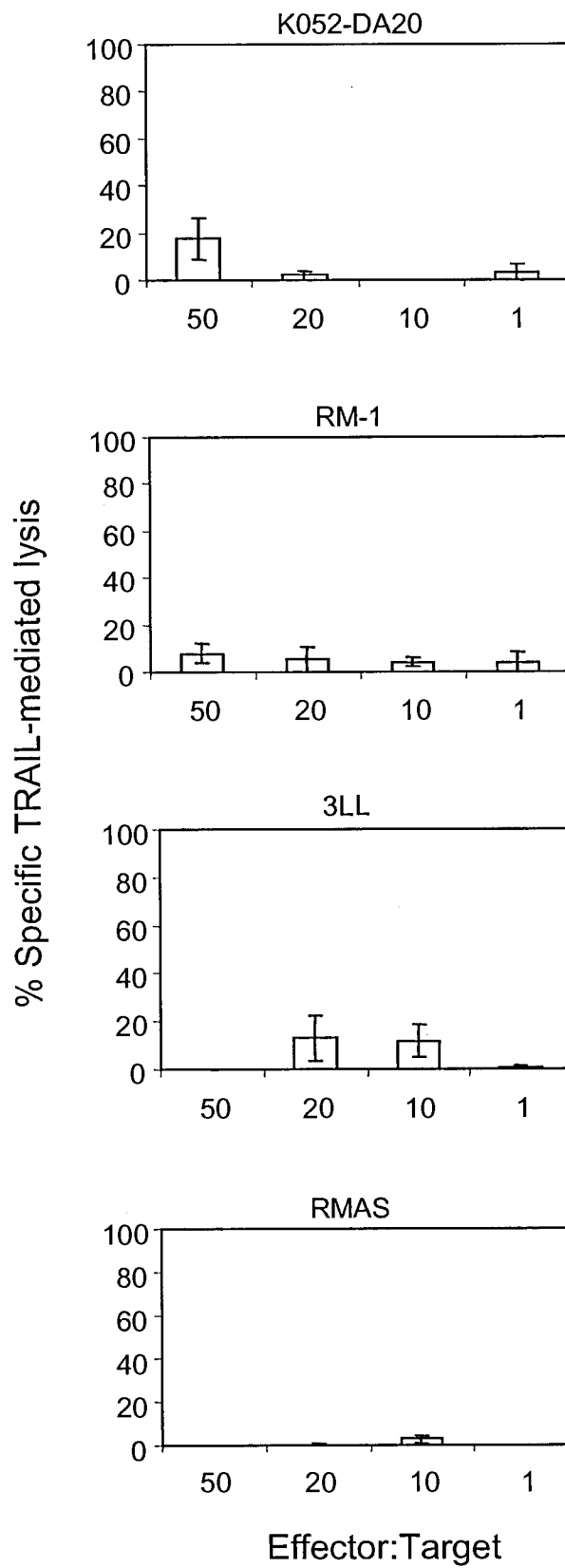


Appendix I. Constitutive TRAIL expression in gene-targeted mice. Liver MNC isolated from adult C57BL/6 mice were stained with antibodies to CD3, NK1.1 and TRAIL or isotype control mAb. Histograms demonstrate TRAIL/isotype control antibody staining on gated NK1.1⁺CD3⁺ cells. Red lines represent staining with anti-mTRAIL mAb, black lines represent staining with isotype matched control mAb. Data are representative of the analysis of 20 C57BL/6, 4 C57BL/6 TNF^{-/-}, 10 C57BL/6 IL-12^{-/-}, 8 C57BL/6 IL-18^{-/-}, 10 C57BL/6 IL-12^{-/-} IL-18^{-/-}, 7 C57BL/6 IL-4^{-/-}, 4 C57BL/6 IFNαR1^{-/-}, and 4 C57BL/6 IFNαR2^{-/-} mice. Liver NK cell TRAIL expression was not statistically different between the strains of mice shown ($P > 0.05$).

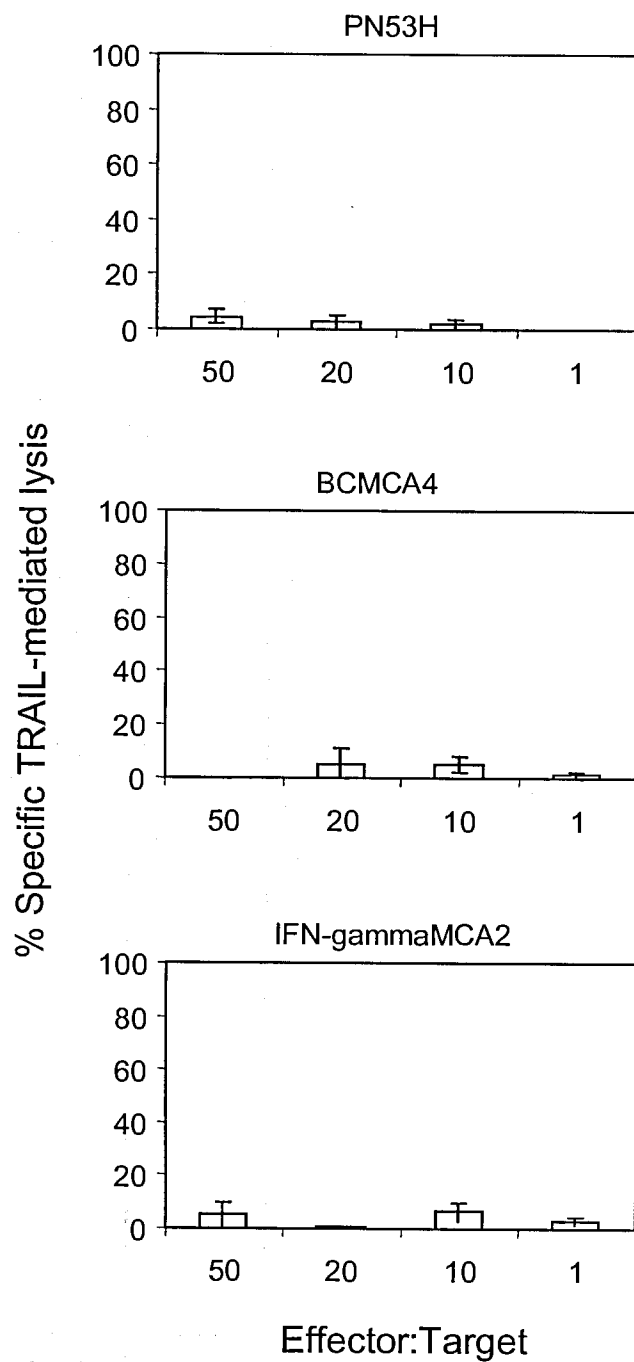


Appendix II. Sensitivity of mouse tumor cell lines to TRAIL-mediated cytotoxicity. The cytotoxic activity of 2PK3-mTRAIL transfectant cells against various mouse tumor cell lines was tested in an 18 h ^{51}Cr release assay. Percent specific TRAIL-mediated lysis was determined by subtracting percent specific lysis by 2PK3 parental cells from % specific lysis by 2PK3-mTRAIL transfectant cells. Data represent mean \pm SEM of 6 4T1.2, 3 DA3, 8 EMT6.5, 3 A20, 20 Renca, 2 KO52-F11, 1 IFN γ MCA3, 1 BCMCA3, 1 EL4, 2 KO52-DA20, 1 RM-1, 1 3LL, 1 RMAS, 1 PN53H, 1 BCMCA4 and 1 IFN γ MCA2 experiment.





Appendix II. Continued.



Mouse (sex)	Age (weeks)	Body weight (g)	Spleen weight (g)	Spleen % BW	aLN weights (g)	aLN % BW
6 (M)	47.10	23.33	0.361	1.54	0.144, 0.183	0.617, 0.784
7 (M)	44.10	30.80	0.380	1.23	0.01, 0.01	0.032, 0.032
36 (M)	50.90	29.20	0.639	2.188	0.049, 0.019	0.168, 0.065
68 (M)	46.60	34.46	0.800	2.322	1.942, 0.023	5.636, 0.067
62 (F)	46.30	35.82	1.004	2.803	0.972, 1.110	2.714, 3.099
1 (F)	40.40	28.43	0.917	3.226	0.322, 0.354	1.126, 1.245
50 (F)	58.90	46.33	0.303	0.654	0.010, 0.001	0.022, 0.015
30 (M)	28.40	22.11	1.890	8.550	0.082, 0.038	0.371, 0.171
34 (M)	41.00	34.50	0.500	1.449	0.012, 0.018	0.035, 0.052
61 (M)	34.70	38.11	1.439	3.776	0.551, 0.368	1.446, 0.966
54 (F)	35.90	18.67	0.144	0.771	0.092, 0.096	0.493, 0.514
27 (F)	43.40	18.01	0.196	1.088	0.110, 0.184	0.611, 1.022
28 (F)	44.70	25.52	0.723	2.833	0.180, 0.175	0.705, 0.686
21 (M)	44.90	31.24	0.769	2.462	0.264, 0.172	0.845, 0.551
43 (M)	38.40	25.30	0.403	1.593	0.085, 0.091	0.336, 0.360
26 (F)	56.60	27.00	0.360	1.333	0.072, 0.021	0.267, 0.077
31 (M)	49.10	32.53	0.603	1.854	0.388, 0.349	1.193, 1.073
49 (M)	41.00	28.94	0.478	1.652	0.349, 0.247	1.206, 0.853
23 (M)	48.60	30.11	0.260	0.864	0.068, 0.070	0.226, 0.232
29 (F)	48.00	27.77	0.700	2.521	0.407, 0.091	1.466, 0.328
40 (F)	44.10	34.75	1.145	3.290	0.857, 0.364	2.466, 1.047
46 (F)	44.00	32.28	0.663	2.054	0.402, 0.398	1.245, 1.233
47 (F)	44.60	33.72	0.718	2.129	0.294, 0.403	0.872, 1.195
48 (F)	45.30	28.81	0.681	2.364	0.093, 0.097	0.323, 0.337
52 (F)	43.60	25.80	0.549	2.128	0.197, 0.16	0.764, 1.543
57 (F)	52.30	25.41	0.761	2.995	0.684, 0.048	2.692, 0.189
63 (F)	51.60	25.69	0.484	1.884	0.396, 0.072	1.541, 0.280
44 (M)	55.90	31.72	1.171	3.692	0.005, 0.005	0.016, 0.016
33 (M)	56.00	33.73	1.639	4.849	0.038, 0.014	0.113, 0.042
59 (M)	55.90	33.91	0.285	0.840	0.136, 0.071	0.401, 0.209
60 (M)	57.30	34.88	0.443	1.270	0.165, 0.112	0.473, 0.321
42 (F)	60.10	29.41	0.316	1.074	2.272, 0.472	7.725, 1.605
37 (M)	67.30	32.82	0.819	2.495	0.620, 0.286	1.889, 0.871
49 (M)	41.00	28.94	0.478	1.652	0.349, 0.247	1.206, 0.853
72 (F)	40.70	32.27	0.904	2.801	0.944, 0.654	2.925, 2.027
16 (F)	56.00	24.26	2.120	8.740	0.046, 0.086	0.190, 0.354
51 (F)	73.40	26.82	0.289	1.078	1.135, 0.570	4.232, 2.125
74 (F)	69.70	27.30	0.283	1.037	0.077, 0.083	0.282, 0.304
73 (F)	46.10					
41 (F)	68.40					
67 (F)	82.00					
17 (F)	38.60					
32 (F)	39.10					
53 (F)	29.00					
56 (F)	32.70					
66 (F)	33.60					
69 (M)	57.60					
38 (M)	50.00					
58 (M)	45.00					
76 (F)	44.00					
39 (F)	52.70					
64 (F)	48.90					
mean	48.38	29.76	0.700	2.397	0.309	1.030
SD	10.92	5.39	0.459	1.768	0.409	1.299
SEM	1.51	0.87	0.075	0.287	0.047	0.149
n	52	38	38	38	76	76

Appendix III. Aged C57BL/6 *gld* mice. Age at death and weights of spleen, aLN and whole body are shown for aged C57BL/6 *gld* mice. Spleen and aLN weights are also shown as a percentage of body weight (BW). Organ and body weights are shown for mice on which a full autopsy was performed.

Mouse (sex)	Age (weeks)	Body weight (g)	Spleen weight	Spleen % BW	aLN weight	aLN % BW
69(M)	89.6	37.86	0.195	0.515	0.001, 0.001	0.003, 0.003
39(M)	91.6	41.16	0.16	0.389	0.01, 0.01	0.024, 0.024
66 (M)	78.9	30.08	0.287	0.954	0.001, 0.001	0.003, 0.003
16 (M)	95.3	33.16	0.991	2.989	0.020, 0.001	0.060, 0.003
42 (F)	46.6	27.28	0.162	0.594	0.046, 0.055	0.169, 0.202
43 (F)	46.3	32.94	0.419	1.272	0.337, 0.317	1.023, 0.962
44 (F)	46.3	25.19	0.482	1.913	0.086, 0.079	0.341, 0.314
59 (M)	44.7	38.15	0.445	1.166	0.112, 0.167	0.294, 0.438
28 (M)	67.4	30.16	0.419	1.389	0.035, 0.009	0.116, 0.030
46 (M)	67.4	30.92	0.424	1.371	0.032, 0.035	0.103, 0.113
19 (M)	77.4	33.23	0.295	0.888	0.018, 0.009	0.054, 0.027
51 (M)	73.6	28.05	0.1	0.357	0.048, 0.010	0.152, 0.036
30 (M)	73.1	22.7	0.25	1.101	0.001, 0.001	0.003, 0.003
31 (M)	72.7	38.68	0.447	1.156	0.001, 0.001	0.003, 0.003
33 (F)	59.6	31.53	0.431	1.367	0.117, 0.223	0.371, 0.707
24 (F)	51.6	25.1	0.298	1.187	0.095, 0.110	0.378, 0.438
26 (F)	61.6	25.55	0.914	3.577	0.049, 0.023	0.192, 0.090
49 (F)	58.3	23.92	0.24	1.003	0.065, 0.060	0.272, 0.251
29 (M)	61.9	34.97	0.264	0.755	0.02, 0.051	0.057, 0.156
52 (F)	72.9	27.32	0.202	0.739	0.162, 0.030	0.593, 0.110
37 (M)	81.4	33.89	0.139	0.41	0.07, 0.01	0.207, 0.030
63 (F)	69.3	31.13	0.571	1.834	1.332, 0.434	4.279, 1.394
56 (F)	68.6	29.02	0.345	1.189	0.062, 0.001	0.214, 0.003
54 (M)	70.4	30.12	0.22	0.73	0.001, 0.001	0.003, 0.003
70 (M)	67.9	27.77	0.291	1.048	0.01, 0.035	0.036, 0.126
40 (F)	80.7	23.03	0.167	0.725	0.039, 0.027	0.169, 0.117
58 (F)	76.7	22.71	0.442	1.946	0.031, 0.014	0.137, 0.062
20 (M)	93.6					
27 (F)	45					
48 (F)	41.9					
50 (F)	40.1					
53 (F)	33.7					
57 (F)	37					
67 (M)	42.7					
41 (M)	46.9					
34 (F)	50.4					
23 (F)	51					
62 (F)	47.7					
32 (F)	63.7					
9 (F)	82.8					
21 (M)	70.1					
61 (F)	64.7					
48 (F)	54.9					
68 (F)	66					
36 (F)	76.4					
22 (M)	79.6					
mean	63.91	30.21	0.356	1.206	0.084	0.276
SD	16.13	5.13	0.211	0.742	0.194	0.621
SEM	2.38	0.96	0.041	0.143	0.026	0.085
n	46	27	27	27	54	54

Appendix IV. Aged C57BL/6 *gld* TNF^{-/-} mice. Age at death and weights of spleen, aLN and whole body are shown for aged C57BL/6 *gld* TNF^{-/-} mice. Spleen and aLN weights are also shown as a percentage of BW. Organ and body weights are shown for mice on which a full autopsy was performed.



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